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SYNTHETIC APPROACHES TO CYCLODEPSIPEPTIDES THE SYNTHESIS OF NORSURFACTIN

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SYNTHETIC APPROACHES TO CYCLODEPSIPEPTIDES. THE SYNTHESIS OF NORSURFACTIN

by

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TO

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THE SYNTHESIS OF NORSURFACTIN

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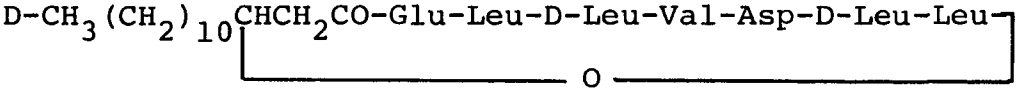
Synthetic strategies are developed for the preparation of cyclodepsipeptides, particularly those possessing amino acid residues with carboxylic acid side chains.

The synthesis of the cyclooctadepsipeptide, norsurfactin, is described as an example of the application of such strategies.



Norsurfactin

A procedure for the formation of tert-butyl esters of β-hydroxy acids using dimethylformamide dineopentyl acetal and tert-butyl alcohol was developed. It was demonstrated that this procedure is superior to previously published methods for the preparation of similar compounds. Steric factors influencing depsipeptide bond formation between amino acids and β-hydroxy acid derivatives were

also investigated.

Improvements in depsipeptide yields using N,N'-carbonyldiimidazole were achieved by systematic modifications in reagent ratios and reaction times, and by using a sodium-imidazole catalyst.

INTRODUCTION

Advancements in the area of peptide synthesis during the past fifteen years have made the preparation of small peptides routine and the synthesis of larger peptides and even small proteins not uncommon. Synthetic procedures for the preparation of cyclic peptides have also been extensively investigated, since many are biologically active, functioning as hormones, fungal toxins, and antibiotics.[1] Procedures for the preparation of depsipeptides (compounds containing both amino and hydroxy acid residues linked through amide and ester bonds) and particularly cyclodepsipeptides, have not been as extensively investigated. Synthetic studies of cyclodepsipeptides have been limited almost entirely to the preparation of compounds containing short, branched-chain α-hydroxyalkanoic acids as part of the ring system, such as valinomycin, the enniatins and their analogs.[2-5] Very little attention has been focused on depsipeptides containing longer chained β-hydroxyalkanoic acids as part of the ring structure. Compounds having these structural features include esperin[6], isariin[7,8], the isarolides[9], peptidolipids NA[10,11], serratamolide[12] and surfactin.[13] To date, only two among this group, isariin[14-16] and serratamolide[17], have been prepared synthetically.

Serratamolide (Figure 1) is the only member of this group with a symmetric, repeating sequence. This structural

feature was exploited in its synthesis by using a unique hydroxyacyl insertion reaction.[17] This reaction has no general applicability in the preparation of the other compounds mentioned.

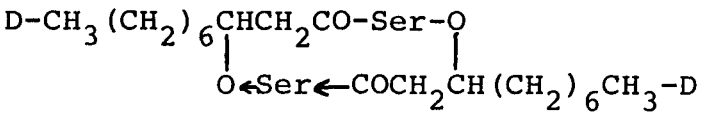


Figure 1. Serratamolide.

Isariin (Figure 2) is a cyclohexadepsipeptide having a structure more representative of the naturally occurring cyclodepsipeptides containing β-hydroxy fatty acid residues.

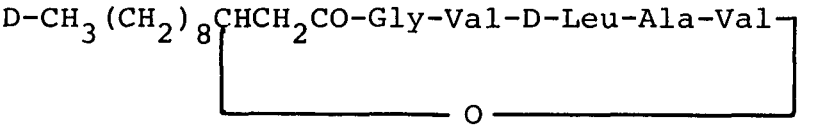


Figure 2. Isariin.

The synthesis of isariin has been accomplished in three different ways, including a solid phase procedure.[14-16] It must be noted, however, that the amino acid residues in isariin have no side chain functionality. This fact greatly simplifies the synthesis. Although the techniques used in the preparation of isariin could be of general utility in the preparation of similar compounds, they are not by them-

selves sufficient, since esperin, peptiodolipids NA and surfactin all contain amino acid residues with third functionality.

There is a need for the development and elaboration of synthetic procedures that are applicable to several classes of cyclodepsipeptides. There is, in fact, a need for a variety of successful procedures, since it has already been firmly established in other areas of peptide synthesis that an extremely successful technique for one compound might fail when dealing with compounds of very similar structure.

In order to investigate and develope synthetic techniques having a broader scope than have been previously reported, syntheses of surfactin, esperin and related cyclodepsipeptides were chosen as long term objectives.

Surfactin (Figure 3) is a cyclooctadepsipeptide containing seven amino and one iso-β-hydroxy fatty acid residue.

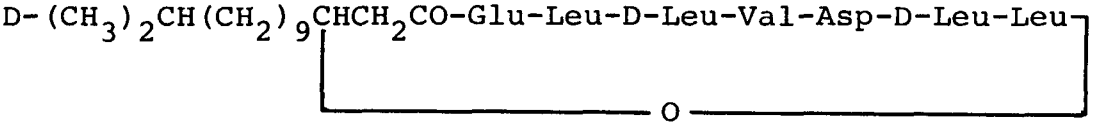
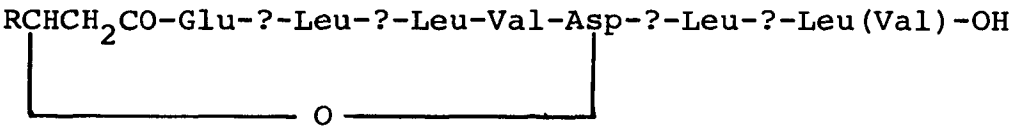


Figure 3. Surfactin (Bacillus subtilis).

Esperin (Figure 4) is also a cyclooctadepsipeptide to which a very similar (but in some respects equivocal) structure has been assigned.



R = n-$C_{12}H_{25}$- (45%)

R = n-$C_{11}H_{23}$- (35%)

R = n-$C_{10}H_{21}$- (20%)

Val C-terminal replacement ∿(30%)

Figure 4. The Structure of Esperin (Bacillus mesentericus).

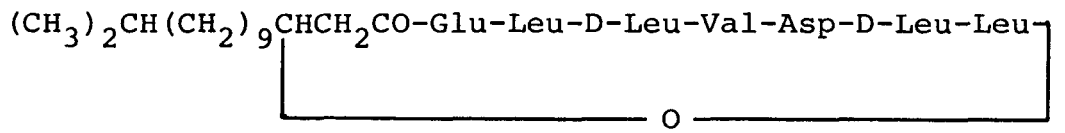
The configurations of the leucine residues are open to question. It is known, however, with reasonable certainty that the D-leucine to L-leucine ratio is one.

The structural similarities and uncertainties of these two compounds, the presence of side chain functionality and their remarkable biological activity make the synthesis of surfactin and esperin an interesting and potentially useful challenge. The development and elaboration of synthetic procedures useful for the preparation of surfactin, esperin and related cyclodepsipeptides is the subject of this thesis.

HISTORICAL

Surfactin

The production of hemolytic factors in the culture medium of certain aerobic sporogenic bacilli was first reported in the 1950's.[18,19] However, it was not until 1970 that Bernheimer and Avigad isolated from B. subtilis a hemolytic compound which they called subtilysin.[20] Earlier, however, Arima and coworkers had isolated and characterized a compound (which they called surfactin) having anticoagulent and antibiotic activity from the culture medium of B. subtilis.[21-27] Subtilysin and surfactin proved to have identical structures (1).



1

Surfactin was isolated from supernatant culture fluids of Bacillus subtilis IAM 1213 by straightforward chemical procedures.[25] The precipitate obtained from acidification of the culture filtrate was converted to a calcium salt. Reacidification gave crude surfactin which was further purified by chromatography on Sephadex G-50 and Sephadex LH-20. Crystalline surfactin was isolated from 50% aqueous acetone after a two month cooling period. A yield of 40-50 mg per liter of culture medium was reported. Surfactin was

obtained as a white crystalline solid, mp 138-140°, $[\alpha]_D$ +40° (c 1, $CHCl_3$) and $[\alpha]_D$ -39° (c 1, CH_3OH).

Surfactin has been shown to be a strong surface active agent, decreasing the surface tension of dilute basic solutions more than twice as much as equivalent concentrations of sodium lauryl sulfate.[22] It is the first compound possessing such strong surface activity to be isolated from microbial origins. High surface activity can be attributed to the ambiphilic nature of the surfactin structure. The extended hydrocarbon chain of the β-hydroxy acid residue and the alkyl side chains of the leucine and valine residues comprise a lipophilic ensemble while the depsipeptide ring and particularly the easily ionizable side chain carboxylic acid moieties of aspartic and glutamic acids are hydrophilic. The surface activity arising from these structural features is more than just an interesting physical property, it is very likely that it contributes to surfactin's unique spectrum of biological activity as well.

Buffered solutions of surfactin were found to be strongly hemolytic.[20] Red cells from a variety of animals were lyzed, but the greatest activity was shown toward erythrocytes from rabbit, man and guinea pig. The presence of divalent cations in the test medium increased the rate of lysis as did rapid chilling. Surfactin's hemolytic activity was found to be inhibited by serum and low concentrations of several phosphatides.

As is typical of many cyclic peptides and depsipeptides, surfactin possesses antimicrobial and bactereolytic activity.[20,25,27] The growth of Streptococcus pyogenes, Caryne diptheriae, and Bacillus megaterium were completely inhibited at dilutions of less than 20 μg/ml and Sarcina lutea and Micrococcus lysodeikicus were partially inhibited at dilutions of 150 μg/ml. Surfactin also displayed lytic activity toward protoplasts and spheroplasts of several Gram-positive bacteria but only one Gram-negative organism (Escherichia coli).

The hemolytic, antimicrobial and bacteriolytic properties of surfactin all in some way result from its ambiphilic structure. Other detergent materials have been found to have related properties.[25] It has been shown that surfactin has a greater affinity for the lipid portion of the cell membrane than for the protein portion. A direct interaction with membrane phospholipids has also been established.[27] Surfactin is known to inhibit other membrane centered processes, such as the utilization of glucose and the synthesis (But not the activity) of alkaline phosphatase.[25] These data suggest that the ambiphilic nature of surfactin allows sufficient solubility in the lipid portion of the membrane so that it can disrupt membrane processes causing a loss of osmotic balance and ultimately cell lysis.

It was anticoagulant activity, not antimicrobial activity that initiated the research which ultimately led to the isolation of surfactin. While searching for substances that would prevent fibrin clot dissolution, Kakinuma and

coworkers determined that the culture broths of several strains of B. subtilis greatly promoted plasmin-catalyzed fibrin deterioration.[21] Initial tests suggested that the anticoagulant activity of surfactin was due to its ability to extend the range of plasmin (a proteolytic fibrin digesting enzyme). It was speculated that surfactin somehow altered the enzyme substrate environment so as to facilitate the activity of the enzyme. However, in more detailed investigations involving an in vitro thrombin-fibrinogen system* (no plasmin present) it was found that a 50% inhibition of thrombin activity occurred at surfactin concentrations of 30 μg/ml. Interestingly, it was found that the proteolytic activity of the enzyme, thrombin, toward fibrinogen leading to the formation of fibrin monomer was not inhibited by surfactin. Sedimentation studies on thrombin-fibrinogen-surfactin systems showed the disappearance of fibrinogen and the appearance of fibrin oligomer but no fibrin polymer or clot. The site of inhibition by surfactin in the clotting system was therefore determined to be the polymerization of fibrin monomer to fibrin polymer. It was proposed that the surfactant nature of the compound enabled

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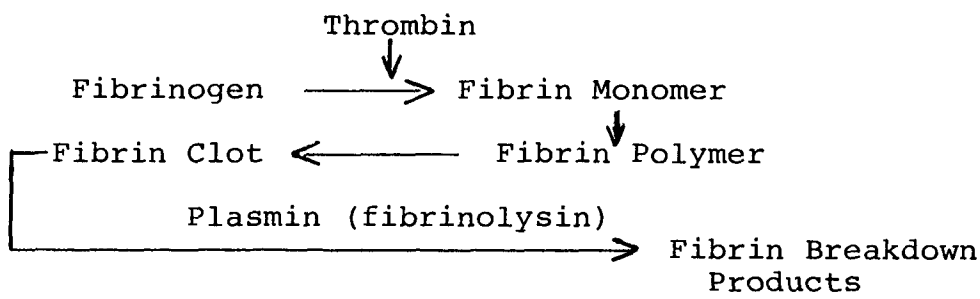
*A simplified scheme of the blood clotting system can be represented by the following diagram:



it to solubilize fibrin oligomers thus preventing further polymerization and precipitation of the fibrin clot. Other detergents are known to dissaggregate and solubilize fibrin polymer.[28] Surfactin is unique in its anticoagulant activity since other anticoagulants, such as the coumarins or heparin, possess little or no *in vitro* activity.*

To date there have been no *in vivo* studies of surfactin's anticoagulant activity. However surfactin has shown a different kind of activity when administered orally to test animals. When surfactin was fed to rats and chickens, a significant lowering of cholesterol levels occurred in both the plasma and liver.[29] Cholesterol levels were lowered by approximately 30% in rats and 50% in chickens when compared to control animals on high cholesterol diets. The activity was found to be specific for cholesterol as the levels of other lipids remained unchanged. This hypocholesterolemic effect was manifested by a 0.3% diet supplement.

More detailed studies showed surfactin inhibited the absorbtion of cholesterol-^{3}H, however, in contrast to the behavior of β-sitosterol (a naturally occurring inhibitor of cholesterol absorbtion[29]) no stoichiometric binding with cholesterol-^{14}C could be observed. Furthermore,

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*For a discussion of other compounds having anticoagulant activity see reference 46a.

studies with surfactin-^{14}C revealed that there was no absorbtion of surfactin in the gastrointestinal tract. Ingested surfactin-^{14}C was completely excreted in the feces. These facts along with the correlation between the critical micelle concentration of surfactin (5-10 μg/ml) and the fact that, in vitro, surfactin-cholesterol interactions begin at surfactin concentrations of 2-20 μg/ml regardless of cholesterol concentration, suggest that the hypocholesterolemic activity of surfactin is a result of physico-chemical interactions. It is likely that the surface active nature of surfactin causes the formation of micelles which selectively include cholesterol and allow it to pass unabsorbed through the gastrointestinal tract.

In summary, in every case of reported biological activity for surfactin there seems to be a direct link to its surface activity. No other surface active agent has been found to possess such a remarkable range of biological activity. This makes the elucidation of structure-activity relationships a complex but potentially significant problem.

The structure of surfactin has been firmly established. Analytical data indicates that the crystalline material isolated from the culture broths of B. subtilis is composed of at least 90% of a material having structure (1).

Acid hydrolysis of the crystalline material resulted in a two phase product. A hydrochloric acid-soluble portion composed of amino acids and an oily, hydrochloric acid-insoluble, portion composed of fatty acids and un-

hydrolyzed surfactin. These results indicated that surfactin was a peptidolipid.

The amino acid sequence of the peptide portion of the molecule was determined by classical techniques.[23] Surfactin was subjected to partial acid hydrolysis and the resulting peptide fragments were separated chromatographically. The amino acid sequences of each of the thirteen peptides obtained were determined using the Edman technique.[30] From sequence overlap data the structure of the linear peptide fragment of surfactin was established as 2 .

Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu*

2

The positions of the D-leucine residues were determined by subjecting the peptide fragments obtained from hydrolysis to the action of D-amino acid oxidase. Amino acid composition was determined before and after enzymatic digestion. Only the peptide fragments containing a D-leucine residue showed the loss of a leucine residue in the amino acid composition after the enzyme treatment. Comparing these results with the previously determined fragment sequence data, the positions of the D-leucine residues were located unambiguously.

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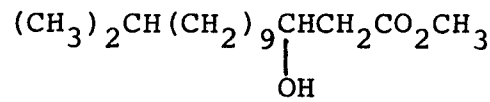
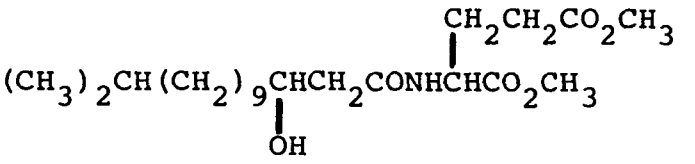
*It should be noted that a small amount of L-isoleucine was also detected indicating minor variations in the peptide sequence.

The determination of the structure of the fatty acid component of surfactin was accomplished as follows.[23] Subjecting crystalline surfactin to methanolysis produced an oily material which was chromatographed on silica gel to give two fractions. The first fraction was composed of a fatty acid methyl ester shown to be greater than 90% pure by GLC. This fatty acid ester was shown to be 13-methyl-β-hydroxytetradecanoic acid methyl ester (3) based on elemental analysis, the infrared spectrum, the nuclear magnetic resonance spectrum and mass spectral evidence. Minor impurities detected during GLC analysis were shown to be esters of C_{13} and C_{14} β-hydroxy acids. These esters represented less than 10% of the methanolysis product.



3

Using the same analytical techniques, a compound found in the second chromatographic fraction was determined to be the dimethyl N-acyl glutamate 4 , confirming that glutamic acid is the N-terminal amino acid residue of the peptide. Thus the entire acyclic structure of surfactin was established as 5 .



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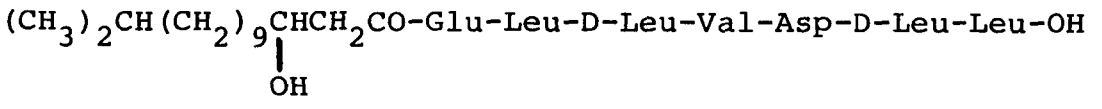


5

It was determined that although surfactin itself was not acylated by treatment with pyridine-acetic anhydride, a compound obtained by mild alkaline hydrolysis of surfactin was easily acylated under these conditions. This suggested that in surfactin the hydroxyl group of the β-hydroxy fatty acid was involved in a lactone bond.

Which of the three available carboxyl groups in the peptide residue formed the lactone bond was determined as follows. When surfactin was subjected to reduction by $LiBH_4$ (only ester bonds being reduced) the amino acid composition of the reduction product indicated the loss of one leucine residue.[31] If surfactin was first treated with diazomethane and then reduced with $LiBH_4$, aspartic acid, glutamic acid and one of the four leucine residues disappeared. When the lactone ring of surfactin was opened prior to reduction by mild alkaline hydrolysis, no change in amino acid composition was observed. These results located the lactone bond between the hydroxyl group of the fatty acid and the

carboxyl group of the C-terminal leucine. Disregarding minor variants, the total structure of surfactin was therefore shown to be 1 .

This structure was later confirmed by mass spectrometry[32] using the techniques of Lederer[33] and Thomas.[34]

It should be noted that the possibility exists that the structure of the active substance in the culture broth might be slightly different than that of the crystalline material. The opening and rearrangement of the lactone bond during isolation and purification could have occurred, however, the high biological activity of the crystalline material makes this unlikely.

Esperin

Isolation of the substance, esperin, from strains of B. mesentericus was first reported in the early 1950's.[35,36] The substance was found to have a very high degree of antibiotic activity toward Mycobacterium tuberculosis.

Available data concerning esperin is far less reliable and precise than that for surfactin. Most of the published reports on esperin are filled with ambiguities, equivocal interpretations of data, and consequently structural assignments that are tenuous. There is even doubt concerning the authenticity of the producing bacterial strain, Bacillus mesentericus.[45] There is, however, sufficient evidence available to indicate that surfactin and esperin are structurally similar. Whether the similarities in structure also manifest themselves as similarities in bio-

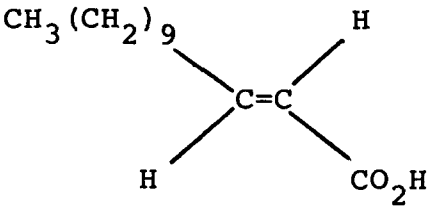
logical activity has yet to be fully elucidated.

The isolation of esperin was accomplished by acidification of the culture broths of B. mesentericus followed by reprecipitation of the resulting solid from methanol-water and acetone-petroleum ether. A solid substance was obtained with a melting point of 235°. The substance inhibited the growth of M. tuberculosis at a dilution of 1:500,000.

It was found that alkaline hydrolysis of esperin followed by acidification yielded a different substance, esperinic acid (mp 105°) which inhibited the growth of M. tuberculosis at dilutions of 1:160,000. This is the only biological activity reported for either material.

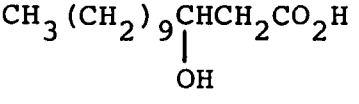
Attempted structural elucidations for esperin and esperinic acid have been plagued with incorrect assignments. The exact structure of these compounds has yet to be unambiguously determined.

The results of the first structural studies were based on analyses of acid hydrolysates.[37] The ether extracts of these hydrolysates were found to contain a material which appeared to be a fatty acid having fourteen to sixteen carbon atoms. The ether insoluble portion contained the amino acids; D,L-leucine, L-aspartic acid and L-valine. The presence of glutamic acid was not reported. More detailed investigation identified the fatty acid as trans-2-tridecenoic acid, (6).[38] No higher or lower homologs of this acid were reported.



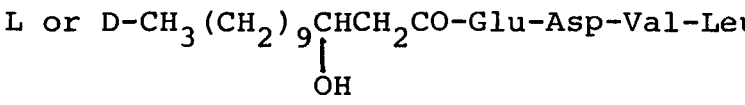
6

It was not until seven years later that the same authors published additional structural data causing a revision of prior interpretations.[38a] Esperin was reported to be a crystalline material (mp 238°) having a molecular weight of 730-800, an optical rotation of -24° (0.66% in methanol) with empirical formula, $C_{39}H_{69}N_5O_{11}$. Analyses of the acid hydrolysates reportedly showed L-glutamic acid as well as L-aspartic acid, L-leucine, D-leucine and L-valine. As before, 2-tridecenoic acid was recovered from the ether soluble portion of the hydrolysates, however, the presence of unsaturation was attributed to the acid catalyzed dehydration of β-hydroxytridecanoic acid (7), the actual fatty acid presumed to be in esperin. The configuration at the asymmetric carbon of the hydroxy acid, along with the possible presence of higher or lower homologs, remained unreported.



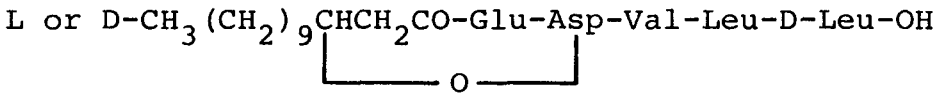
7

Based on the results of complete and partial acid hydrolysis, spectral data and a carboxypeptidase C-terminal amino acid determination (the significance of which will be discussed later), the structure of espernic acid (produced by mild alkaline hydrolysis of esperin) was proposed as 8 .



8

Successive hydrazinolysis, Curtius rearrangement and hydrolysis of esperin transformed the aspartic acid residue to α,β-diaminopropionic acid indicating that the β-carboxyl group of the aspartic acid residue participated in the lactone bond. Based on these data the structure of esperin was proposed as 9 .



9

Citing reasons such as "some of the data of the Japanese authors did not seem very reliable" and "compounds of such unusual structure containing a cyclotridepsipeptide system have not been met previously in nature", a Soviet group decided to test the validity of structures 8 and 9 by total synthesis. In two separate and unambiguous syn-

theses, compounds having structures 8 and 9 were prepared. Separate diastereomeric compounds containing both D- and L-β-hydroxytridecanoic acid residues were prepared in the case of 8 , however in the preparation of 9 only the synthetic intermediate containing the L-β-hydroxy acid could be converted to the cyclic product. All of the compounds synthesized were totally void of antibiotic activity and their physical properties were completely different from those of the natural substances. The proposed structures of esperin (9) and esperinic acid (8) were therefore shown to be incorrect.

Faced with this new evidence, the original investigators undertook a new structural investigation.[6] Collaborating with D. W. Thomas and using his newly developed technique involving mass spectrometry of permethylated peptide derivatives[34], some of the ambiguities concerning the structure of esperin were clarified. From the mass spectral data it was determined that the fatty acid formation in the acid hydrolysates was a mixture of components and not a single compound. The presence of 2-tridecenoic acid along with its C_{14} and C_{15} homologs now indicated the following three β-hydroxy acids were present in esperin in the percentages indicated in Figure 5. It should be noted that β-hydroxytridecanoic acid (7) was the only previously reported component and it comprises only 20% of the actual mixture.

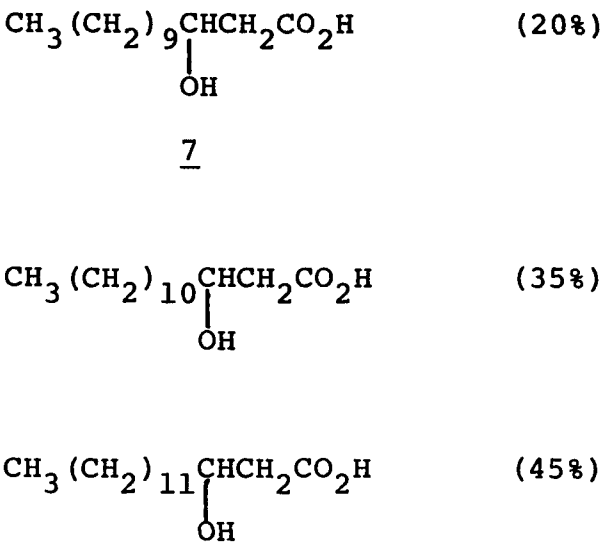


Figure 5. The β-Hydroxy Fatty Acids Present in Esperin.

Additional mass spectral data revealed the entire amino acid sequence. It was determined that esperin contained, not a pentapeptide residue as previously thought, but the heptapeptide 10 . However, the peptide portion of esperin was not homogeneous; L-valine appeared as a C-terminal variant in approximately 30% abundance. Mass spectral data could not provide any information about the configuration of the amino and hydroxy acid residues.

Glu-?-Leu-?-Leu-Val-Asp-?-Leu-?-Leu(L-Val)

10

Based on all the available data the structure of the esperin complex may be represented as shown in Figure 4. It is evident that several uncertainties remain in the structure of esperin. Of major concern are the configurations of the leucine residues. It is known that there are two D- and two L-leucine residues in the molecule, however, which residue is of which configuration is unknown. It should be recalled that in the original incorrect structure 9 the C-terminal leucine was assigned the D-configuration while the penultimate leucine residue was determined to be L. The assignments were based on the fact that when esperin and esperinic acid were subjected to carboxypeptidase (an enzyme which successively hydrolyzes L-amino acids from the C-terminal end of peptides and proteins) no hydrolysis took place. The investigators concluded from this that the C-terminal leucyl residue was of the D-configuration (therefore blocking enzymatic degradation). The presence of a D-leucine at the C-terminal end of the peptide would be expected to inhibit carboxypeptidase hydrolysis, however, the fact that no hydrolysis occurred is insufficient evidence to warrant the assignment of the D-configuration. It has been shown that in the case of an N-acyl dipeptide, the presence of a D-amino acid in the penultimate position greatly inhibited the carboxypeptidase hydrolysis of a C-terminal L-residue.[42] In another case concerning the structural determination of isariin (Figure 2), a proposed structure based on the assumption that carboxypeptidase

inhibition was caused by the presence of a D-amino acid was proven incorrect.[14,43] Chemical synthesis verified a correct structure based on the assumption that a penultimate D-amino acid inhibited the enzyme. Clearly then, the assignment of the D-configuration to the C-terminal residue of 9 is not warranted. Since inhibition of carboxypeptidase could be expected if the penultimate leucine residue was of the D-configuration and the C-terminal leucine residue was of the L-configuration (it should be noted that this is the same sequence that appears in the last two C-terminal residues of surfactin, Figure 3).

The assignment of the D-configuration to the C-terminal leucine looks even more doubtful when one considers that only L-valine (no D-valine) was found to be present in the acid hydrolysates, where as, both D- and L-leucine were identified. Considering the fact that valine apparently appears as a C-terminal variant in 30% abundance, it seems unlikely that L-valine would replace D-leucine in a compound derived from an enzyme mediated biosynthesis. Seemingly the only valid conclusion that can be drawn from the carboxypeptidase data is that at least one of the leucine residues in the C-terminal leucylleucine couplet is of the D-configuration. There is no information available concerning the configurations of the internal leucine residues. All of the questionable configurations could be determined by the D-amino acid oxidase technique used in surfactin.[22]

The configurations of the β-hydroxy acid residues have also not been determined. It should be noted, however, that the β-hydroxy acid components of isariin, peptidolipids NA, serratamolide, surfactin and the related peptolide, viscosin[46] have all been shown to have the D-configuration.

Another uncertainty in the structure concerns the C-terminal valine variant. Does valine appear in equal abundance with each fatty acid homolog or does it occur exclusively with only one or two of the fatty acids? In the case of the peptidolipids NA, it was shown that the single variant residue occurred in the presence of only one of the three homologous β-hydroxy fatty acids.[11]

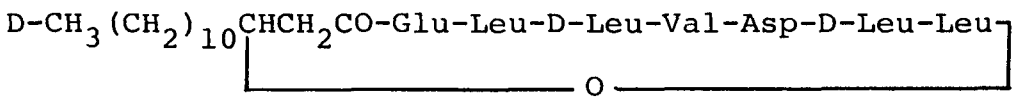
Although there are many uncertainties surrounding the structure of the esperin complex it is evident that esperin and surfactin are structurally very similar. They may, in fact, have identical peptide sequences. The β-hydroxy fatty acid portion of the compounds are undeniably different, surfactin being composed primarily of an iso-fatty acid while the esperin complex is composed of a homologous series of normal fatty acids. The positions of the lactone bonds in the isolated crystalline materials are also different. However, since the isolation procedures for both substances differ, a rearrangement of the lactone bond in one or both compounds during workup, albeit unlikely, cannot be ruled out.

The true similarities between the two substances will not be known until the structure of esperin is completely elucidated. The esperin complex should be resolved into its components, possibly using high pressure liquid chromatography, and the structure of each component should be analyzed with emphasis on fatty acid composition, sequence and configuration of amino acids and variation of C-terminal residues. However, unavailability of the producing strain, B. mesentericus, makes this type of analysis impossible. Alternatively, compounds having the most probable structures could be synthesized and their physical and biological properties compared with those reported for the natural materials. Once the structure of the experin complex is determined, a comparison of the full spectrum of biological activity of both esperin and surfactin could provide information concerning structure-activity relationships for both compounds.

When examining both esperin and surfactin as potential target compounds in the development of synthetic techniques, obvious difficulties appear even before specific synthetic strategies are considered. In the case of the esperins, the uncertainties in structure demand that a number of variants be synthesized. A synthetic problem of this magnitude could be more easily dealt with when suitable synthetic techniques for the rapid preparation of compounds in this class of cyclodepsipeptides have been developed.

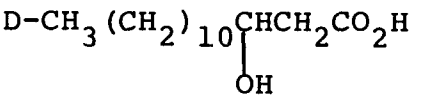
Surfactin is a more appropriate compound for initial study. Its structure is unambiguous and its variety of biological activity should make verification of a synthetic sample relatively easy. The greater availability of natural surfactin for comparative purposes also must be considered. However, the presence of an iso-β-hydroxy fatty acid in the structure of surfactin presents additional complications in the synthesis. Before dealing with the synthesis of the depsipeptide portion of the structure, the preparation of optically pure D-13-methyl-β-hydroxytetradecanoic acid would have to be accomplished. Although several acceptable routes to β-hydroxy acids are available, including a procedure that leads directly to optically active compounds[44], suitable synthetic precursors to D-13-methyl-β-hydroxytetradecanoic acid are not available and would themselves have to be prepared. The preparation of sufficient quantities of D-13-methyl-β-hydroxytetradecanoic acid to begin the synthesis, if not difficult, would at least be time consuming.

In order to avoid this added complication and focus directly on the preparation of the depsipeptide portion of the molecule, a compound having structure 11 , labeled norsurfactin, was chosen as the initial synthetic objective.



11

The only structural difference between this compound and surfactin is the absence of the iso-methyl group on C_{13}. Suitable precursors to this normal β-hydroxy acid, D-β-hydroxytetradecanoic acid (12), are commercially available and the time required to prepare sufficient quantities of optically pure material should be greatly reduced.



12

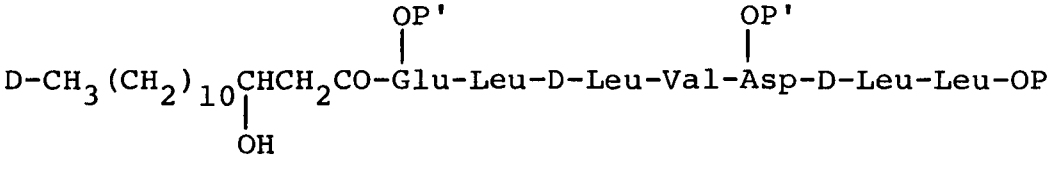
It is not unreasonable to assume that the absence of the methyl group would have little affect on the biological properties of surfactin. There is no loss or change of chirality involved and the biological activities of surfactin seem to be a result of its surface activity which also should not be greatly affected by such a change.

Techniques developed in a successful preparation of norsurfactin could be used both directly in the preparation of surfactin (when sufficient iso-β-hydroxy acid is available) and with slight modifications in the synthetic studies of the esperins.

RESULTS AND DISCUSSION

The direction of a synthetic strategy leading to the preparation of norsurfactin (11) is necessarily determined by the nature of the cyclization step. There are two possible options; the formation of the depsipeptide ring via a cyclization forming the single lactone bond, or the formation of the depsipeptide ring via a cyclization forming one of the seven peptide bonds. Both techniques have advantages and disadvantages.

The preparation of cyclodepsipeptides by cyclizations through lactone bonds has been reported. Several syntheses of the actinomycins and their analogs have been carried out using this technique.[47-50] Implementation of a lactonization cyclization strategy for norsurfactin would require the preparation of the acyclic peptidolipid intermediate 13.



P,P' = carboxylic acid protecting groups

13

The synthesis of this intermediate could be carried out using straightforward peptide coupling techniques.[51] Protecting groups for the side chain carboxylic acid moieties of aspartic and glutamic acid and the C-terminal leucine

residue would have to be carefully chosen, however, the protecting group on leucine (P) must be able to be removed selectively, leaving the protecting groups on the aspartic and glutamic residues (P') intact. The P' groups can be removed only after the cyclization has been completed. Base sensitive protecting groups (e.g., methyl or ethyl esters) could not be chosen for P' since subjecting the cyclized product to an alkaline medium required for their removal would certainly cleave the lactone bond. The choice of both carboxylic acid protecting groups would have to be co-ordinated with the selection of N-terminal amino protecting groups to be used in a fragment or stepwise build up of the peptide chain. Although the hydroxyl group of the β-hydroxy acid residue would not have to be protected if suitable coupling reagents were used[39], it is evident that the choice of the other protecting groups mentioned would have to be considered carefully.

Synthesis of acyclic intermediates similar to 13 have been reported in the literature, but usually only as part of a short-cut method of structure proof. In studies of synthetic approaches to derivatives of esperin[40] and isariin[43], completely deprotected, acyclic β-hydroxy acyl peptides somewhat similar to 13 have been prepared. The same derivatives could, in principle, be obtained from the natural cyclodepsipeptides by mild alkaline hydrolysis provided correct structural assignments have been made. Comparison of the synthetic compounds with hydrolytic pro-

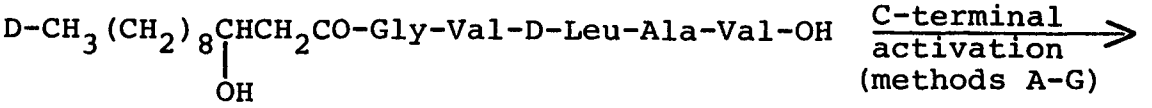
ducts from the natural material short-circuited the need for a total synthesis of the complete cyclodepsipeptides. The previously proposed structure of esperin was thus shown to be incorrect and a suspected structure for isariin was confirmed.

A structure proof is not of primary importance in this study. The structure of surfactin is based on relatively firm evidence. In addition, the fact that there is a structural difference (albeit a very minor one) between norsurfactin and natural surfactin would, nevertheless, make the validity of a comparison between synthetic and naturally derived intermediates questionable.

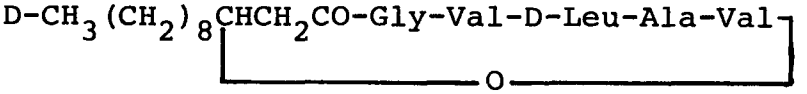
Other disadvantages in a strategy based on the preparation of the intermediate 13 become obvious when one more closely examines the literature on lactone bond cyclizations. A carboxyl group usually requires a much higher degree of activation for the formation of ester or lactone bonds than it does for the formation of amide or lactam bonds. Generally acid chlorides[52], benzenesulfonic anhydrides[41] or highly activated esters[49,53] are used to form depsipeptide ester bonds. If such techniques were to be used to lactonize 13, the risk of racemization of the activated C-terminal leucine residue would be great. Also there are reports that even the aforementioned, and several other, highly activating techniques have failed to promote the formation of lactone cyclodepsipeptide bonds.[54-56] Particularly pertinent is a report on the attempted synthesis of isariin (Figure 2).

The cyclization of an intermediate similar to 13 was attempted via the formation of a lactone bond (Figure 6). None of the desired cyclized product, isariin, could be detected as a result of using any of the procedures listed in Figure 6, even when high pressure liquid chromatography was used to analyze the reaction mixtures. On the basis of these reports, a lactonization strategy for the preparation of norsurfactin would not appear to be a promising approach.

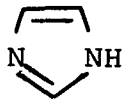
The closure of the depsipeptide ring of norsurfactin via formation of one of the amide bonds is the alternate choice. Even at first glance, the availability of a greater variety of proven peptide bond forming techniques tends to make this route appear more attractive than lactonization. In principle, the cyclization coupling step could be accomplished at any of the seven amide bonds in norsurfactin. However, even though a lesser degree of carboxyl activation is needed for amide bond formation, racemization can occur with all activation procedures and is of constant concern to peptide chemists.[62,62a] It becomes even more significant in cyclizations since the activated carboxyl group is usually present in solution for longer periods of time than in routine peptide couplings. It survives longer because of the conditions used to maximize the yield of cyclic product. The longer the activated group remains in solution the greater the chance of racemization of the C-terminal residue.



Isariic Acid



Isariin

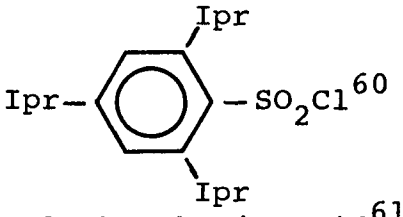
A. CH_3COCl/  [49] ⟶ no product isolated

B. $SOCl_2$ [53] ⟶ "

C. N,N'-dicyclohexylcarbodiimide [57] ⟶ "

D. N,N'-carbonyldiimidazole [58] ⟶ "

E. Dimethylformamide-dineopentyl acetal [59] ⟶ "

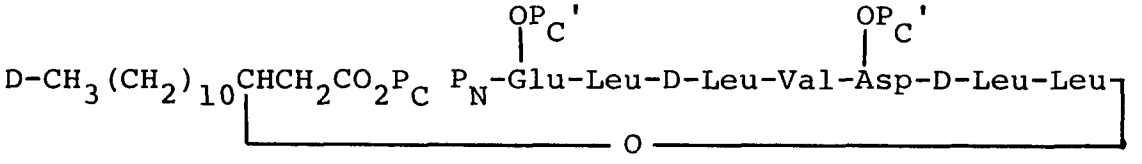
F.  [60] ⟶ "

G. Polyphosphoric acid [61] ⟶ "

Figure 6. Attempts to Lactonize Isariic Acid to Isariin.[43]

Another consideration in designing an amide bond cyclization strategy is the steric hindrance encountered between the two residues involved in the ring-forming step. One would expect lower yields when residues with bulky side chains (e.g., leucine or valine) are involved.[63]

Considering the above facts, there is a single most favorable position for the site of amide bond formation leading to cyclic product. A cyclization between the carboxyl group of the D-β-hydroxytetradecanoic acid residue and the amino group of the glutamic acid residue becomes the logical choice. This strategy would require the preparation of the acyclic intermediate 14.



14

P_C, P_C' = carboxylic acid protecting groups.

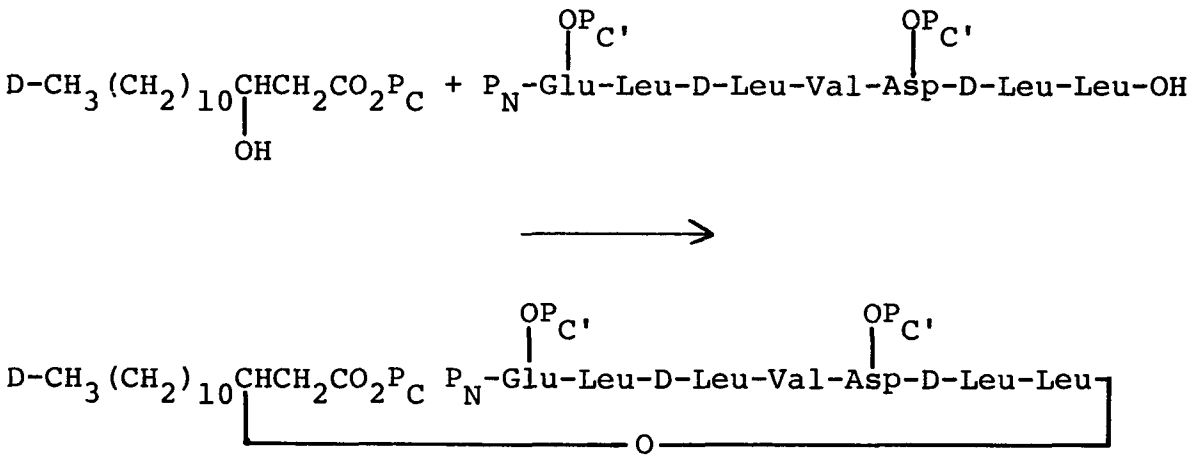
P_N = amino protecting groups.

By attempting a cyclization between the β-hydroxy acid and glutamic acid residues, the risk of racemization is greatly diminished. The center of chirality in the β-hydroxy acid residue is β to the activated carboxyl group and would not be expected to undergo racemization. Steric hindrance should also be less of a problem since there would be no alkyl side chain α to the activated carboxyl group.

The preparation of intermediate 13 could be developed along one of two routes. The heptapeptide chain with protected third functions and N-terminal group could be prepared and then esterification of the C-terminal leucine would give product 14 (Figure 7). Alternatively the ester bond could be formed first between the carboxyl protected D-β-hydroxytetradecanoic acid derivative and a suitably protected leucine derivative. The peptide chain could then be lengthened by stepwise addition of amino acid derivatives, or by fragment coupling to produce 14 (Figure 8).

In at least one case the former procedure (Figure 7) gave unsatisfactory results[15] (very low yields for the peptide esterification step) while there have been several reports of satisfactory results in similar systems using the second approach (Figure 8).[14-16,41] Therefore, the development of a synthetic strategy for norsurfactin based on the preparation of intermediate 14 as shown in Figure 8 was judged to be the more attractive alternative.

Many methods have been developed for the protection of amino, carboxylic acid and other functional groups in peptides and amino acids. However, the requirements imposed by the structure of intermediate 14 and a strategy such as outlined in Figure 8 greatly restrict the protecting groups. Since the ester bond is to be formed initially, any protecting groups requiring alkaline conditions for removal are eliminated. The groups protecting the carboxylic acid moiety of the β-hydroxy acid (P_C in Figure 8) and the groups protect-

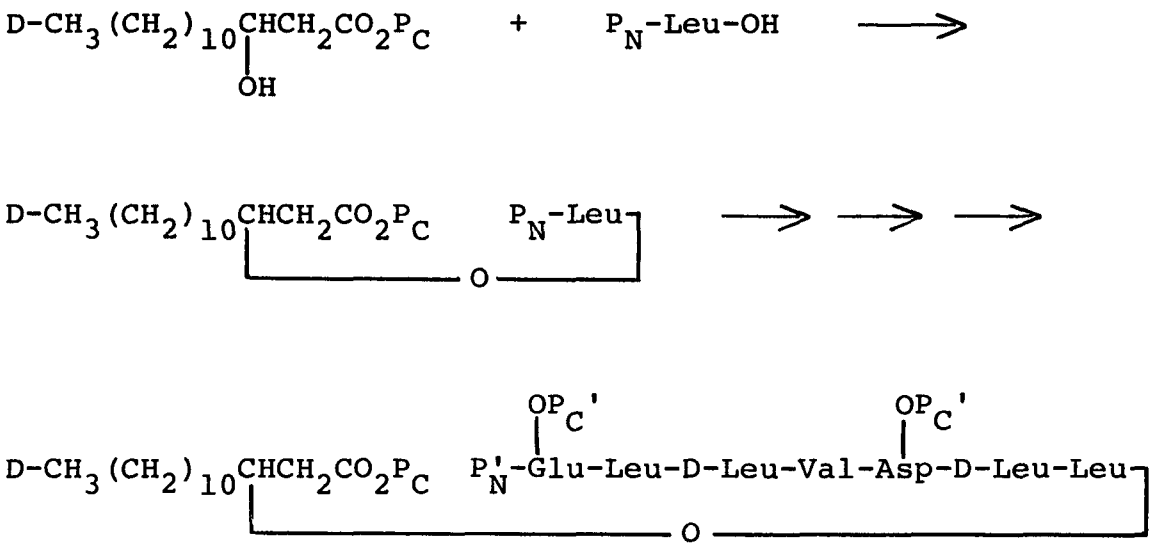


14

$P_{C'}$, $P_{C'}$ = carboxylic acid protecting groups

P_N = amino protecting groups

Figure 7. The Preparation of Intermediate 14 via Esterification of the Heptapeptide.



14

P_C, P_C' = carboxylic acid protecting groups.

P_N, P_N' = amino protecting groups.

Figure 8. The Preparation of Intermediate 14 by Initial Formation of the Ester Bond Followed by Lengthening of the Peptide Chain.

ing the side chain carboxylic acid moieties of the aspartic and glutamic acid residues (P_C' in Figure 8) must be stable to conditions that will remove the N-terminal protecting groups used during the build up of the peptide. The P_C' protecting groups must also be stable to the removal conditions of both the P_C and P_N' protecting groups that must be removed prior to cyclization.

There is a greater flexibility in the choice of methods for the protection of the N-terminal positions of the C-terminal leucine residues encountered in the first two steps of the peptide chain elaboration sequence. The essential limitation to be considered for these residues is that the group used (P_N) must be removable under conditions that will leave the P_C group intact (Figure 9). However, when the protected aspartic acid residue is incorporated into the depsipeptide chain, the requirements for N-terminal protection are more restrictive. For example, the N-terminal protecting group on the aspartic acid residue (and on all subsequent residues) must be removable under conditions that will leave both the P_C and P_C' groups intact (Figure 10).

A final consideration is that the protecting groups on the third functions of the aspartic and glutamic acids must withstand the cyclization step and be removable under conditions that won't effect the peptide or lactone bonds in the cyclized product.

Keeping in mind all of these limitations, a specific synthetic strategy based on the one outlined in Figure 8 may be formulated.

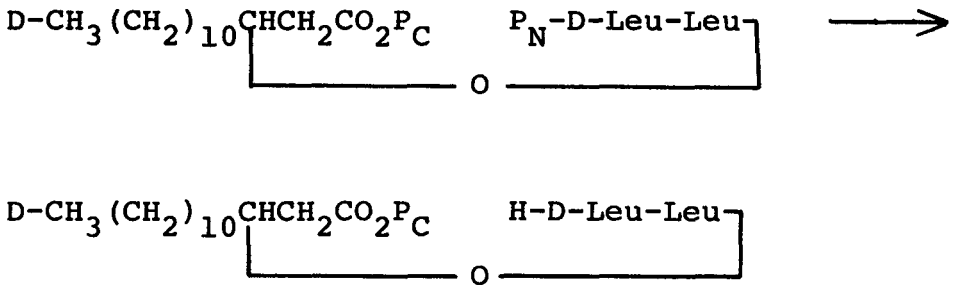


Figure 9. The Removal of the N-terminal Protecting Group on D-leucine, During Elaboration of the Peptide Chain.

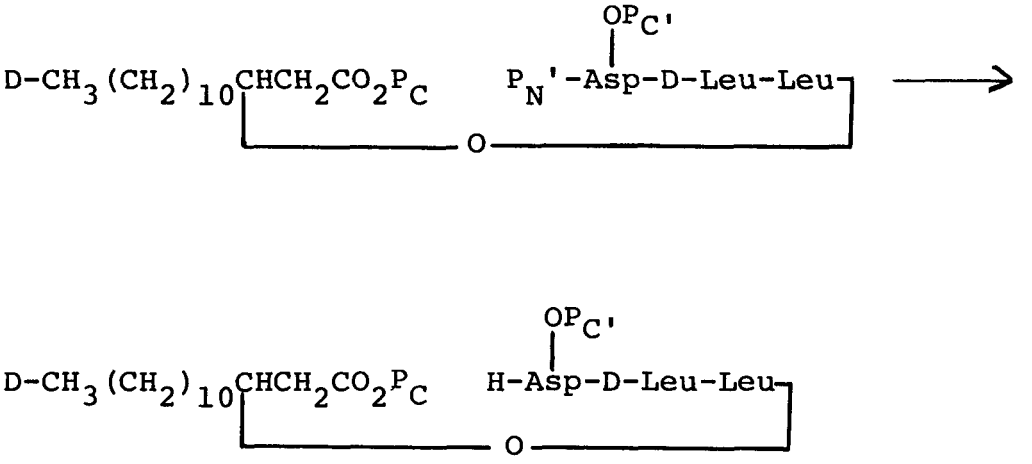


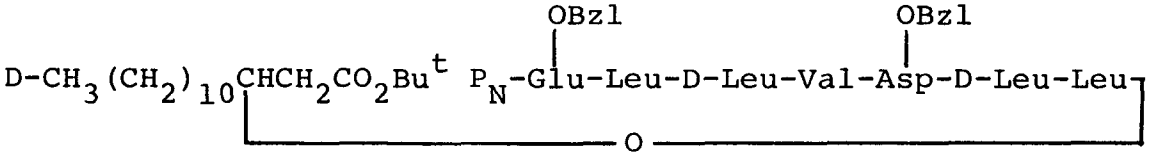
Figure 10. Removal of the N-terminal Protecting Group from Aspartic Acid.

Of the numerous methods reported for the protection of carboxylic acid groups in amino acids and peptides, esterification has proven to be the most useful.[64,65] The use of methyl or ethyl esters in the norsurfactin synthesis is eliminated because the alkaline conditions needed for their removal would affect the latent lactone bond. However, tert-butyl, benzyl and substituted benzyl esters are alternative carboxyl acid protecting groups. Conditions used for their removal are mild and non-alkaline making them suitable for the synthesis of norsurfactin.

The utility of tert-butyl esters (OBu^t) for peptide synthesis was first demonstrated by Anderson[66] and Trachner.[67] tert-Butyl esters of amino acids are easily prepared by an acid catalyzed reaction with isobutylene. The esters thus formed are stable to alkaline and hydrogenolytic conditions but may be easily removed under mildly acidic conditions that will not affect peptide bonds or most other protecting groups.

The benzyl ester (OBzl) was first introduced to peptide chemistry by Bergmann.[68] Benzyl esters of amino acids and peptides are typically prepared by acid catalyzed esterification using benzyl alcohol. The most convenient method for their removal is catalytic hydrogenation, although, benzyl esters may be removed under acidic and alkaline conditions also. They are, however, stable to the mildly acidic conditions needed to remove tert-butyl esters.

Both benzyl and tert-butyl esters could be used in this synthesis. If, for example, in the case of intermediate 14, group P_C were a tert-butyl ester and the P_C' groups were benzyl esters (14a) then the criteria established for carboxylic acid protecting groups would be satisfied. The tert-butyl ester on the β-hydroxy acid residue could be removed with mild acid leaving both benzyl esters intact. After cyclization the benzyl esters could be removed hydrogenolytically without disturbing either the lactone or peptide bonds.



14a

Having justified the selection of suitable carboxylic acid protecting groups, attention can now be focused on N-terminal amino protecting groups. Protection of the amino groups on the amino acid and peptide derivatives required for the synthesis must be compatable with the methods chosen for carboxylic acid protection and with the coupling procedures used.

Methods for the protection of amino groups in peptide synthesis are even more numerous than those available for carboxylic acid protection.[69,70] By far the most useful and most important class of N-terminal protecting groups are the substituted urethans. Bergmann and Zervas introduced the

benzyloxycarbonyl protecting group (Z) in 1932[71] and it is still one of the most often used urethan protecting groups.

Benzyloxycarbonyl amino acids are obtained almost exclusively from benzyloxycarbonyl chloride and the corresponding amino acid in aqueous alkaline solution (Figure 11).

Benzyloxycarbonyl amino acids are stable to mild acid and alkaline hydrolysis conditions. Many other acid labile protecting groups can be removed easily in the presence of a benzyloxycarbonyl group. The Z group is generally cleaved by catalytic hydrogenation, although it can be removed cleanly by acidolysis as well.

The success of the benzyloxycarbonyl group led to extensive investigations in search of other useful urethan protecting groups. Several alkyl and aryl urethans, other than the Z group, have been used for amino protection, however, only one, the tert-butoxycarbonyl group (BOC)[72] has found frequent use.

The BOC group has been used extensively in peptide synthesis and has been the major method of N-terminal protection in solid phase peptide synthesis.[73] The most convenient method for the attachment of the tert-butoxycarbonyl group has been to react an amino acid or peptide with tert-butoxycarbonyl azide (Figure 12). The tert-butoxycarbonyl group is stable to alkaline conditions as well as catalytic hydrogenolysis but is easily removed under mildy acidic conditions. It can be removed selectively in the presence of many other protecting groups including benzyloxycarbonyl

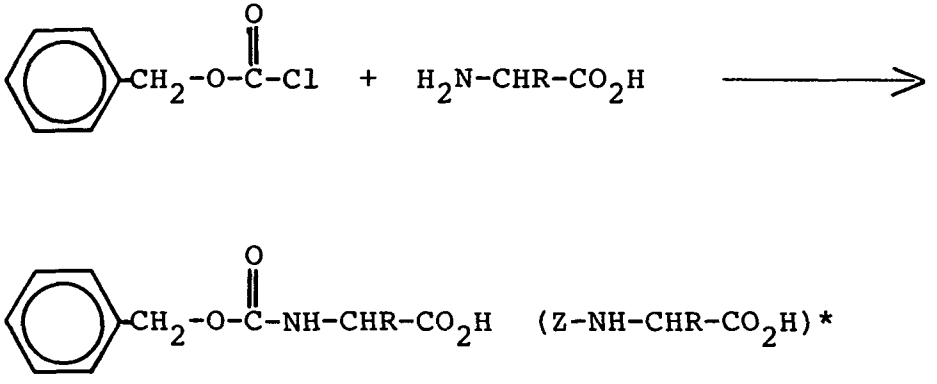


Figure 11. The Preparation of Benzyloxycarbonyl Amino Acids.

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*The abbreviation now generally accepted for the benzyloxycarbonyl group is Z, although it was originally called a carbobenzyloxy or carbobenzoxy group and was abbreviated Cbo.

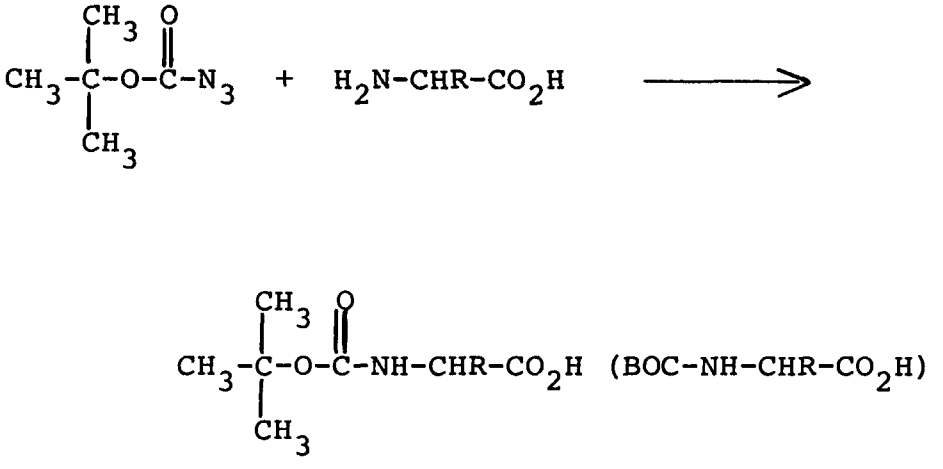


Figure 12. The Preparation of tert-Butoxycarbonyl Amino Acids.

groups and benzyl esters. However, tert-butyl esters are generally not stable to the conditions required for the removal of tert-butoxycarbonyl groups.

The use of benzyloxycarbonyl and tert-butoxycarbonyl groups for amino protection parallels the use of benzyl and tert-butyl esters for carboxylic acid protection. The selection of amino protecting groups for the synthesis of norsurfactin was, therefore, dependent upon the scheme of carboxylic acid protection. Having chosen a tert-butyl ester for the protection of the carboxylic acid at the C-terminal end of depsipeptide 14a, the use of a tert-butoxycarbonyl group for amino protection during elongation of the peptide was eliminated, since selective removal of one group in the presence of the other is not usually possible. However, the tert-butoxycarbonyl group could be used in the singular case of the N-terminal glutamic acid residue (i.e., P_N = BOC in 14a) since the P_N group and the tert-butyl ester must both be removed prior to cyclization.

The benzyloxycarbonyl group could be used in the early steps of elongation of the peptide chain. The amino groups of the first two leucine residues could be protected with Z groups which could be removed selectively by catalytic hydrogenolysis without disturbing the tert-butyl ester on the β-hydroxy acid residue (Figure 13).

The choice of amino protecting groups for the next four amino acids becomes more complex. Once the β-benzyl aspartate residue is incorporated into the peptide chain

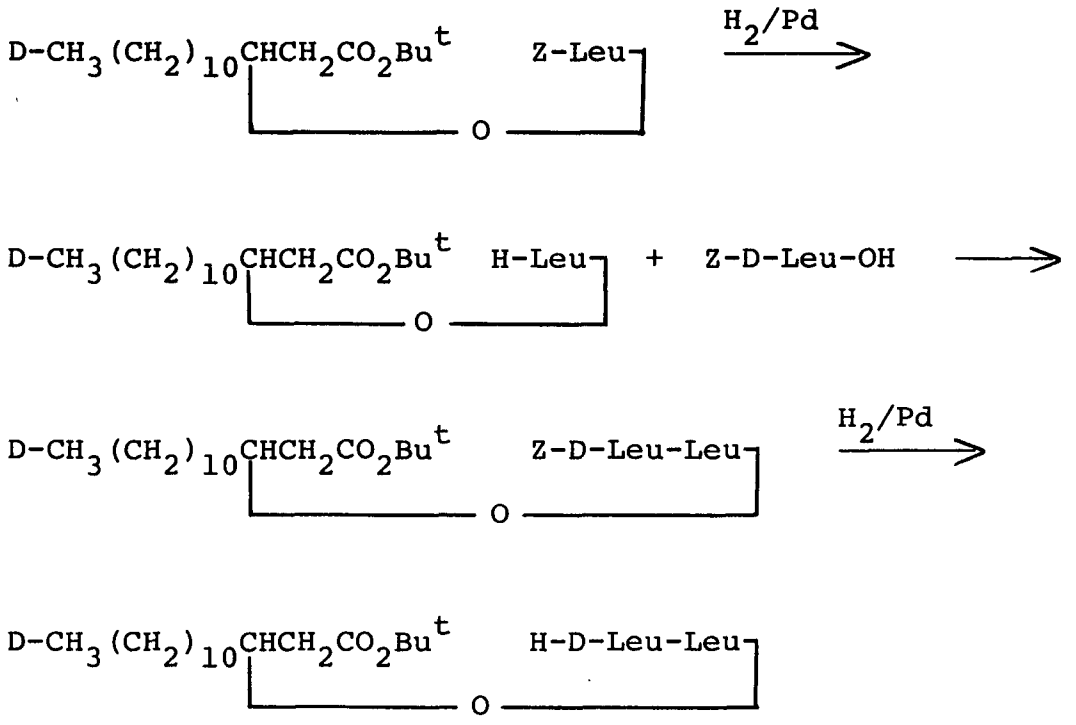
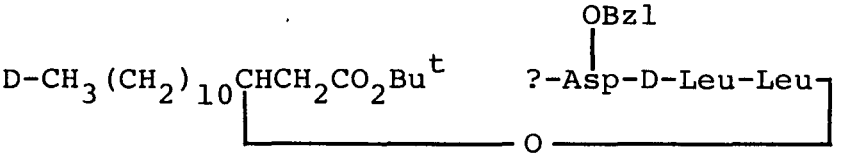


Figure 13. The Use of the Benzyloxycarbonyl Group (Z) for Amino Protection of the First Two Leucine Residues.

(15), neither a tert-butoxycarbonyl nor a benzyloxycarbonyl group can be used for N-terminal protection. Conditions for removal of either of these two groups would also remove one of the carboxylic acid protecting groups. A protecting group must be chosen that can be removed without disturbing the benzyl and tert-butyl esters. There are relatively few amino protecting groups that meet these criteria.



15

The 2-p-biphenyl-2-propyloxycarbonyl group (Bpoc-) introduced by Sieber and Iselin[75] is a urethan type protecting group that does qualify. Bpoc amino acids are prepared in the same manner as tert-butoxycarbonyl derivatives, by reaction with the appropriate azide (Figure 14). The rate of acidolysis of the Bpoc group is 3000 times greater than that for a tert-butoxycarbonyl group and a Bpoc group can, therefore, be removed selectively without effecting either tert-butoxycarbonyl groups or tert-butyl esters.[76] However, the instability and difficulties encountered in the preparation of the 2-p-biphenyl-2-propyloxycarbonyl azide along with the instability of the Bpoc amino acids are disadvantages that have limited the application of this method.

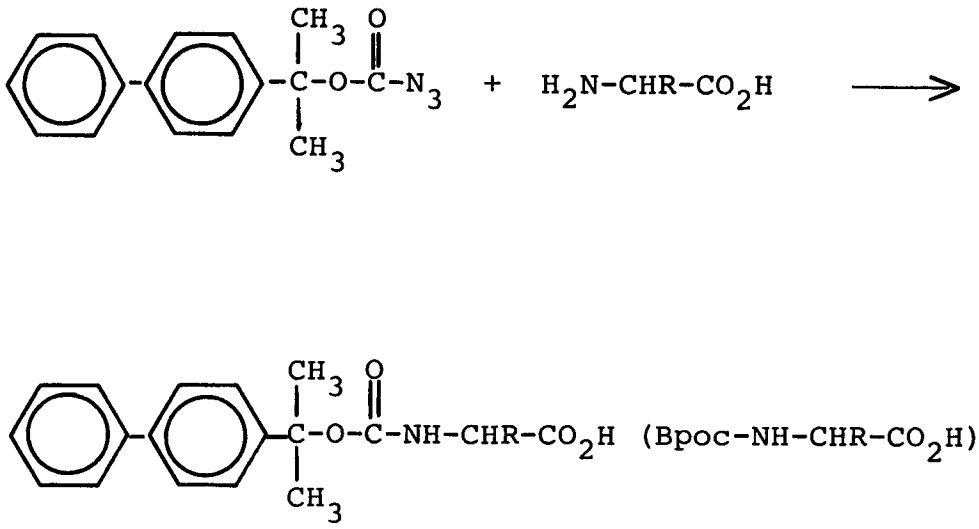


Figure 14. The Preparation of 2-p-Biphenyl-2-propyloxy-carbonyl Amino Acids.

Another amino protecting group that meets the limitations imposed by the presence of both benzyl and tert-butyl esters is the o-nitrophenylsulfenyl group (NPS-) introduced by Zervas.[77] NPS amino acids are formed by reaction of the amino acid with o-nitrophenylsulfenyl chloride in aqueous alkaline solution (Figure 15). By treatment with low concentrations of HCl in non-polar solvents, a o-nitrophenylsulfenyl group can be removed selectively, without disturbing tert-butoxycarbonyl groups and tert-butyl esters.[78] NPS groups can also be removed by treatment with various thiols.[79] Unlike the 2-p-biphenyl-2-propyloxycarbonylgroup, there are no serious problems with stability of either o-nitrosulfenyl chloride or the resulting protected amino acids. Because of its greater stability, the variety of suitable removal techniques and the fact that the o-nitrophenylsulfenyl group has been used as a protecting group for the preparation of similar intermediates[41], it appeared to be a more promising choice for the protection of the amino group of aspartic acid and the remaining residues in the norsurfactin synthesis (Figure 16).

Once suitable protecting groups have been chosen, a complete synthetic strategy may be developed. A synthetic scheme involving the stepwise elongation of the depsipeptide followed by a cyclization via formation of an amide bond is summarized in Chart I.

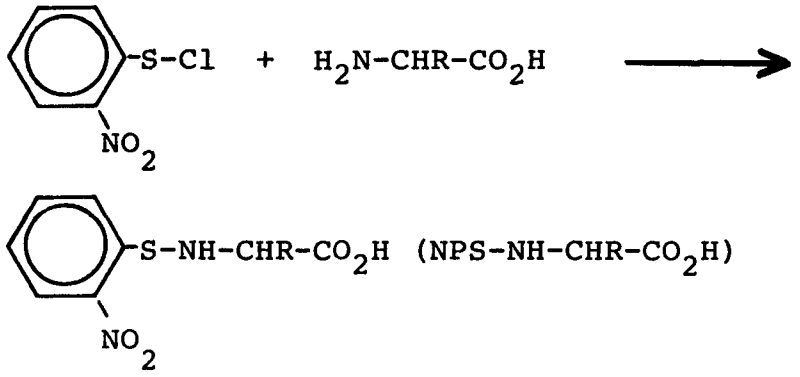


Figure 15. The Preparation of o-Nitrophenylsulfenyl Amino Acids.

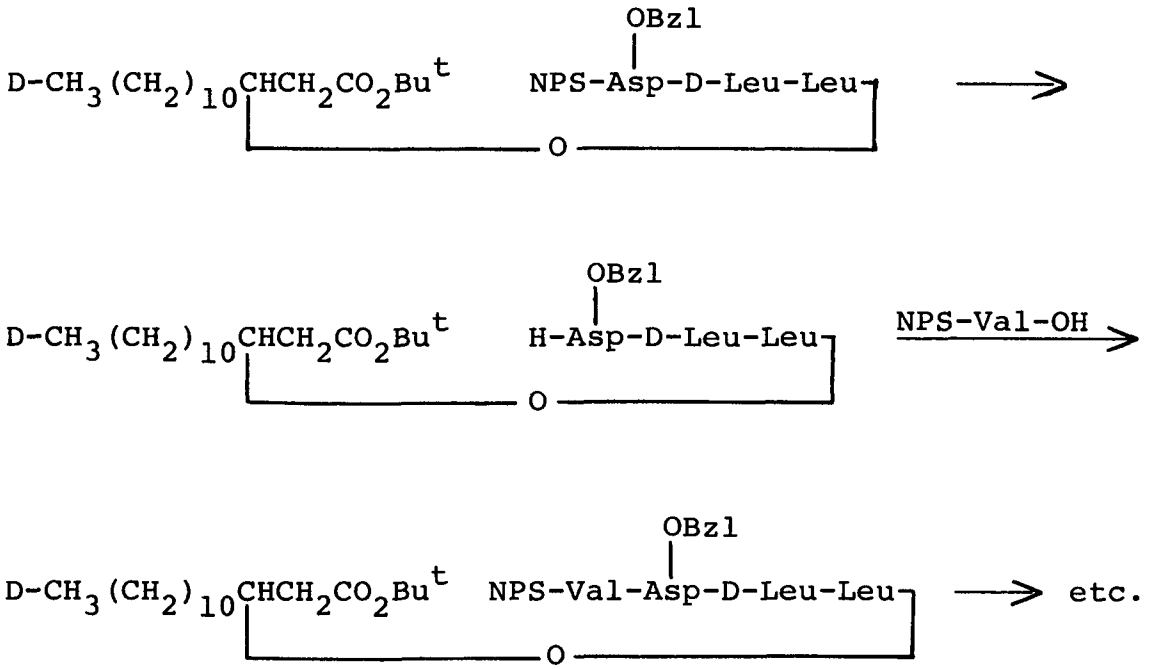
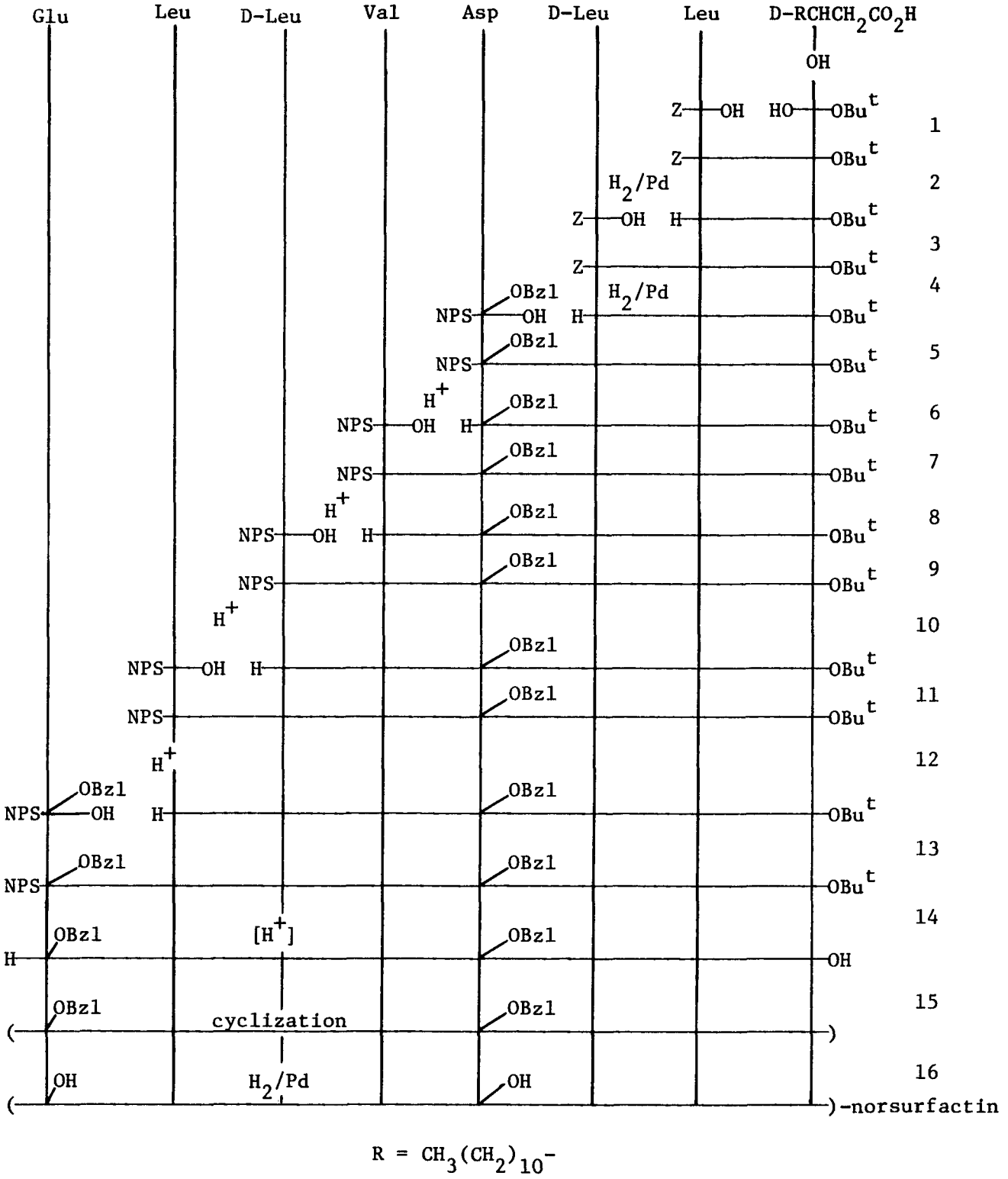


Figure 16. The Use of the o-Nitrophenylsulfenyl Group for Amino Protection.



$R = CH_3(CH_2)_{10}-$

Chart I. A Synthetic Strategy for the Stepwise Synthesis of Norsurfactin, Variation A.

Step 1 involves the formation of the ester bond between benzyloxycarbonyl-L-leucine and tert-butyl-D-β-hydroxytetradecanoate to give a protected didepsipeptide. In step 2 the benzyloxycarbonyl group is removed from the didepsipeptide by catalytic hydrogenation. The free N-terminal didepsipeptide thus obtained is coupled to benzyloxycarbonyl-D-leucine (step 3) to afford a protected tridepsipeptide. The benzyloxycarbonyl group is again removed by catalytic hydrogenation (step 4) and the free N-terminal tridepsipeptide is coupled to o-nitrophenylsulfenyl-β-benzyl aspartate (step 5). The selective removal of the o-nitrophenylsulfenyl group by treatment with low concentrations of HCl in a non-polar solvent is to be accomplished in step 6. Consecutive addition of o-nitrophenylsulfenyl protected amino acid residues and selective cleavage of the o-nitrophenylsulfenyl groups (steps 7-13) would lead to a completely protected acyclic octadepsipeptide derivative. Acidolysis of this derivative under more vigorous conditions (step 14) would remove both the o-nitrophenylsulfenyl group from the N-terminal glutamic acid residue and the tert-butyl ester from the C-terminal D-β-hydroxytetradecanoic acid residue. The free N- and C-terminal octadepsipeptide can then be cyclized (step 15) and the remaining benzyl ester protecting groups removed by catalytic hydrogenation (step 16) to give norsurfactin.

The scheme just described formed the basis for initial synthetic studies. The first consideration was the preparation of suitably protected amino and β-hydroxy acid residues.

Racemic β-hydroxytetradecanoic acid (12) was prepared in 65% yield using Rathke's modification of the Reformatsky reaction (Figure 17).[80,81]

Dodecanal (16) was allowed to react with ethylbromoacetate (17) in the presence of zinc dust in a mixed tetrahydrofuran-trimethylborate solvent system (1/1, v/v) at room temperature. The resulting racemic ethyl-β-hydroxytetradecanoate (18) was saponified with a solution of potassium hydroxide in ethanol to afford the desired (±)-β-hydroxytetradecanoic acid (12) in 65% overall yield.

Racemic β-hydroxytetradecanoic acid had been previously resolved on a small scale by Ikawa using D-amphetamine as the resolving agent.[82] A more feasible large scale resolution was developed using (+)- and (-)-α-methylbenzylamine as resolving agents. (-)-D-β-Hydroxytetradecanoic acid of greater than 96% optical purity was obtained in 14% yield after several recrystallizations of the (-)-α-methylbenzylamine salt. (+)-L-β-Hydroxytetradecanoic acid was obtained in a similar fashion using (+)-α-methylbenzylamine.

A tert-butyl ester was chosen as the protecting group for the carboxylic acid moiety of the β-hydroxy acid residue. Preparation of tert-butyl esters of similar β-hydroxy acids have been reported. For example, as part of early attempts to synthesize esperin, tert-butyl-β-hydroxytridecanoate (both enantiomers) was prepared by the reaction of tert-butylbromide with the silver salt of β-hydroxytridecanoic acid (Figure 18). A complicated column chromatographic procedure involving

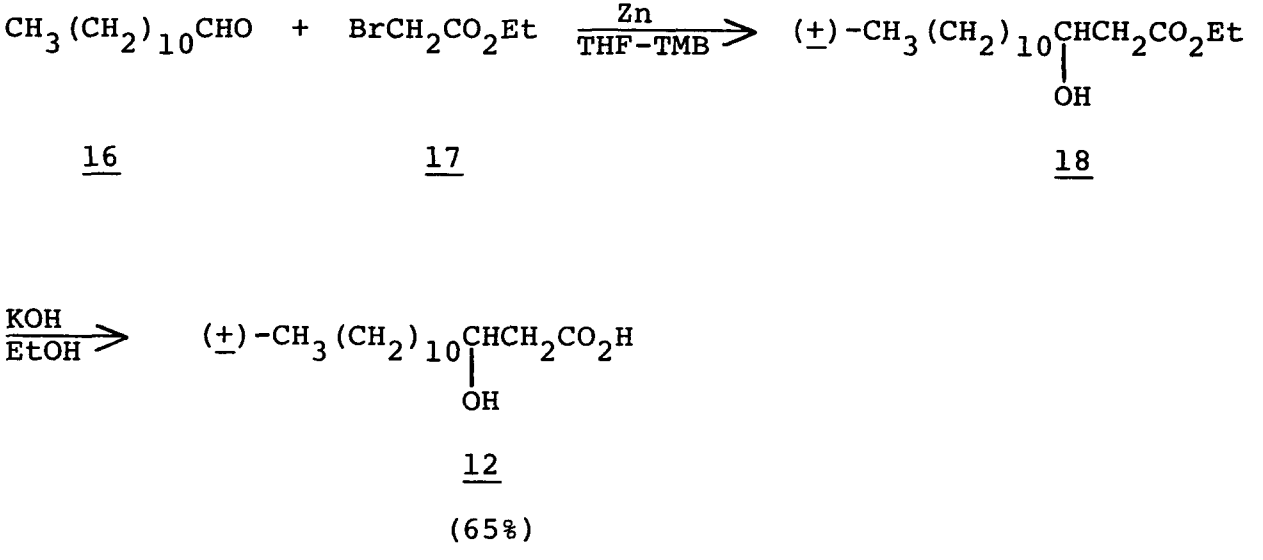


Figure 17. The Preparation of Racemic β-Hydroxytetradecanoic Acid.

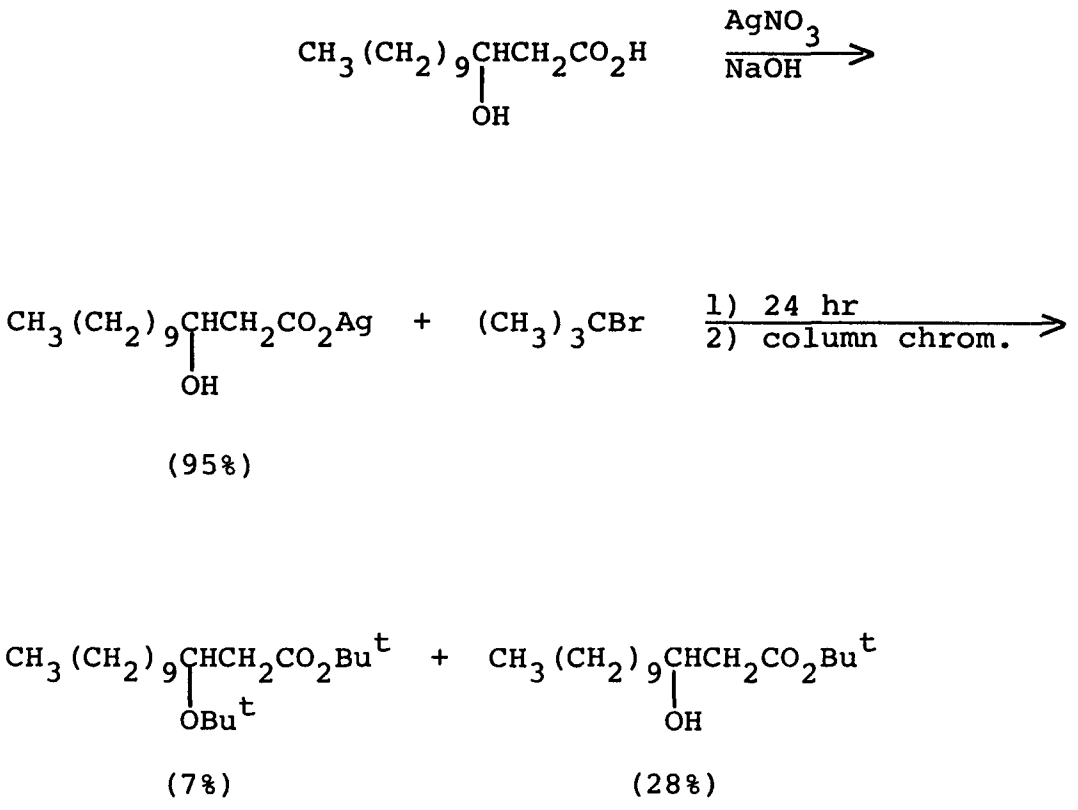


Figure 18. The Preparation of tert-Butyl-β-hydroxy-tridecanoate.[41]

gradient elution with a benzene-hexane solvent system was required to separate the desired tert-butyl-β-hydroxytridecanoate from β-tert-butoxytridecanoic acid tert-butyl ester which was also formed in the reaction. This problem and the low overall yield of the reaction made this an unattractive route to tert-butyl-D-β-hydroxytetradecanoate.

In the synthesis of isariin, tert-butyl-β-hydroxydodecanoate was prepared via a different route (Figure 19).[15]

The acetoxy derivative was first prepared by reaction of D-β-hydroxydodecanoic acid with acetic anhydride in pyridine. Acid catalyzed esterification with isobutylene gave tert-butyl-D-β-acetoxydodecanoate. Saponification of this intermediate afforded the desired tert-butyl-D-β-hydroxydodecanoate in 60% overall yield. Despite the increased overall yield in this sequence compared to that illustrated in Figure 18, it is still relatively unattractive because of the number of steps involved. The methods described in Figures 18 and 19 were explored, but were abandoned in favor of a new approach.

A major problem in the preparation of tert-butyl esters of β-hydroxy acids is the prevention of tert-butyl ether formation at the hydroxyl group during the esterification. It was found that this side reaction could be avoided by using dimethylformamide dineopentyl acetal to mediate the esterification of the β-hydroxy acid with tert-butanol (Figure 20).

Racemic tert-butyl-β-hydroxytetradecanoate (20) was prepared from D,L-β-hydroxytetradecanoic acid (12) and dimethylformamide dineopentyl acetal (19) in tert-butanol.

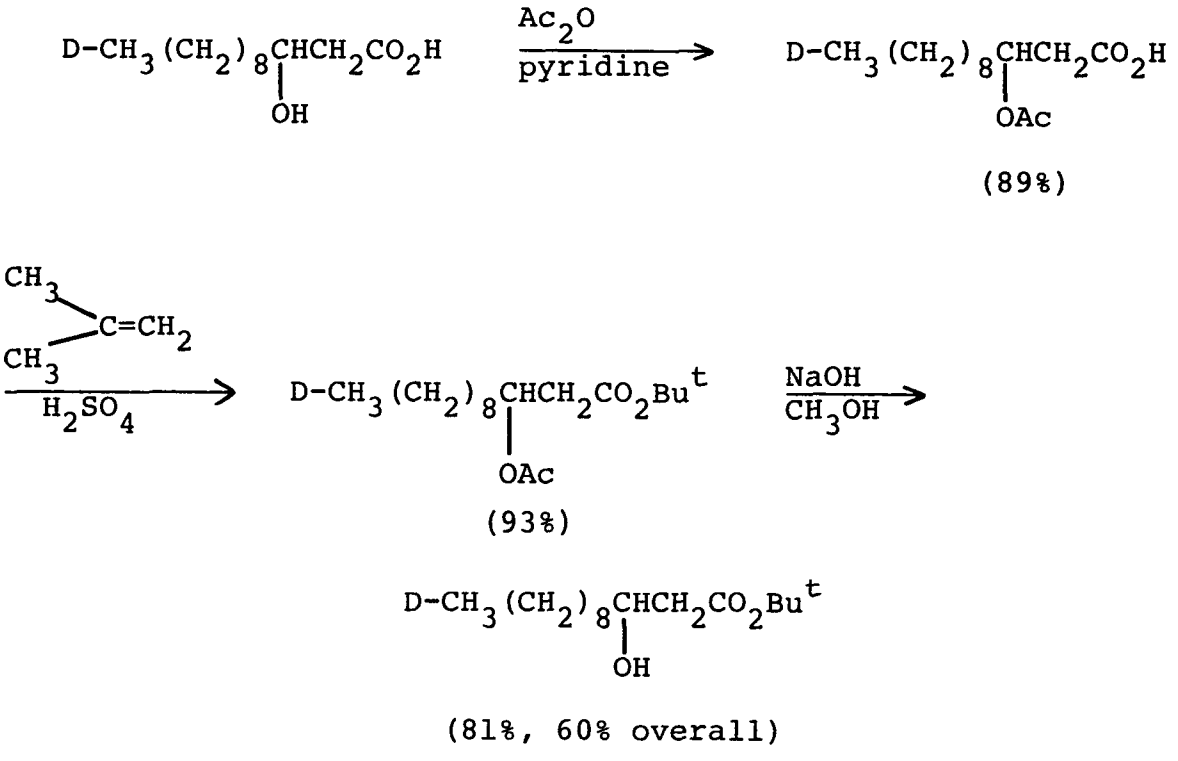


Figure 19. The Preparation of tert-Butyl-D-β-hydroxydodecanoate.[15]

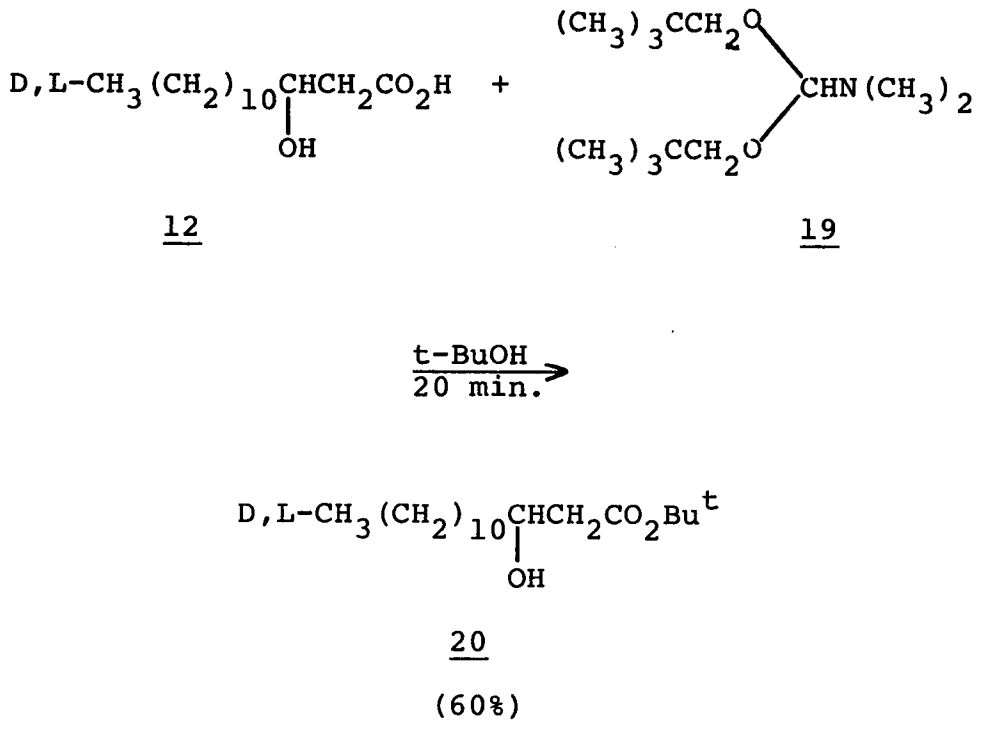


Figure 20. The Preparation of Racemic tert-Butyl-β-hydroxy-tetradecanoate.

The product was isolated from the reaction mixture in 60% yield using dry column chromatography. This method for the preparation of tert-butyl esters of β-hydroxy acids seems to be superior to those previously reported (Figures 18 and 19).

Benzyloxycarbonyl-L-leucine and all other benzyloxycarbonyl amino acid derivatives used in this study were prepared in the manner shown in Figure 11.[84]

The formation of the ester bond between benzyloxycarbonyl-L-leucine and tert-butyl-D-β-hydroxytetradecanoate (step 1 in Chart I) is a key step in the norsurfactin synthetic strategy (Figure 21). There are several reports in the literature on the esterification of amino acids with hydroxy acids (depsipeptide bond formation). Of particular significance to this work was the benzenesulfonic anhydride procedure (using benzenesulfonyl chloride) employed by Ovchinnikov[41] to accomplish the esterification of α-nitrobenzyl-benzyloxycarbonyl-aspartate with tert-butyl-D-β-hydroxytridecanoate (Figure 22).

Another procedure for depsipeptide bond formation which has, in many cases, proven to be superior to mixed anhydride techniques is the use of N,N'-carbonyldiimidazole (CDI). This reagent converts the carboxylic acid group of the amino acid to a highly activated imidazolide which then reacts with the hydroxy component in the reaction to form an ester (Figure 23).

Gisin and Merrifield[85] reported the use of N,N'-carbonyldiimidazole for the esterification of tert-butoxy-

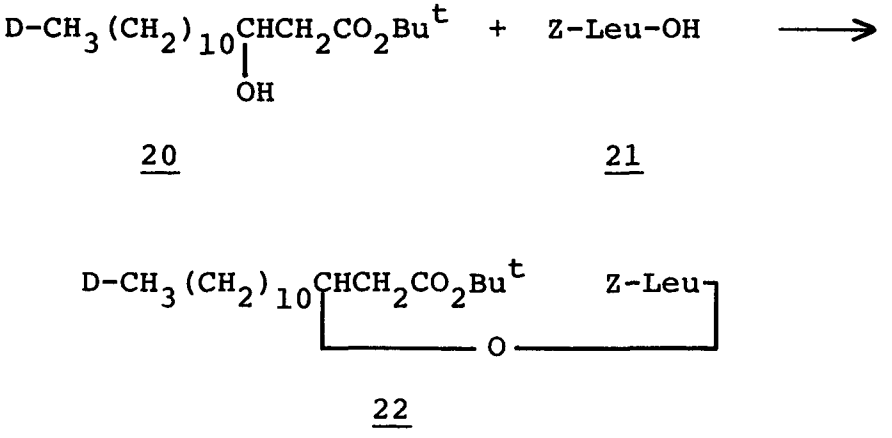


Figure 21. The Formation of the Ester Bond in Step 1 of the Norsurfactin Synthesis.

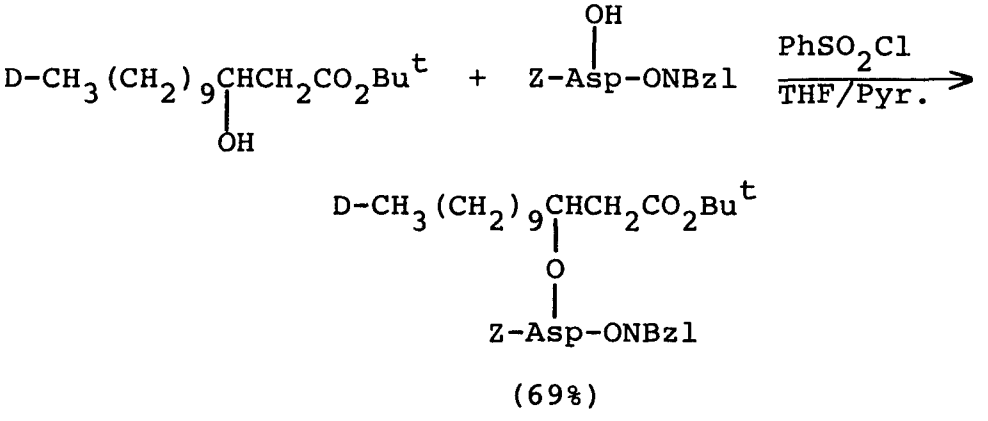


Figure 22. The Formation of a Depsipeptide Bond Using Benzenesulfonyl Chloride.[41]

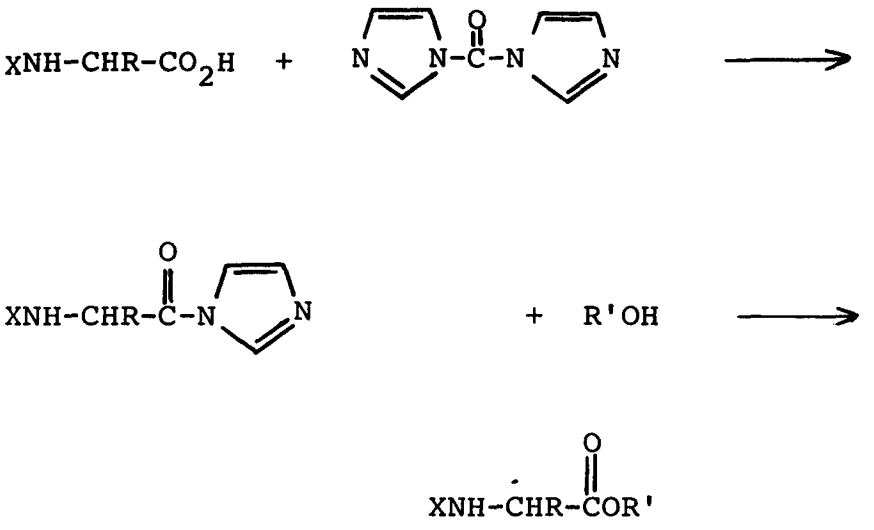


Figure 23. The Use of N,N'-Carbonyldiimidazole for Amino Acid Esterification.

carbonyl-L-valine with benzyl lactate (Figure 24).[85] Although Figure 24 illustrates the esterification of an amino acid using an α-hydroxy acid derivative, N,N'-carbonyldiimidazole has been used with β-hydroxy acid derivatives also (Figure 25).[14]

In the present work, formation of the ester bond in norsurfactin (step 1, Figure 21) was first attempted using racemic tert-butyl-β-hydroxytetradecanoate. Both the N,N'-carbonyldiimidazole and benzenesulfonic anhydride activation techniques were studied. The results of several reactions are listed in Figure 26.

Under the conditions shown in Figure 26, N,N'-carbonyldiimidazole in either methylene chloride or tetrahydrofuran failed to afford any of the desired product 22. The reactions were monitored by Gas Liquid Phase Chromatography; little or no disappearance of the tert-butyl-β-hydroxytetradecanoate could be detected. When the reactions were worked up only starting materials were recovered. Several attempts to form esters using benzenesulfonyl chloride also failed to produce any of the desired product.

In view of the success of these procedures in previously published reports, our results seemed to be anomalous. The lack of success, might be attributed, at least in part, to steric hindrance. The steric bulk of the isobutyl side chain on the leucine residue and of the tert-butyl ester on the β-hydroxy acid residue might conspire to inhibit esterification. It was found that when benzyloxycarbonylglycine (23) was substituted for benzyloxycarbonyl-L-leucine (21) in

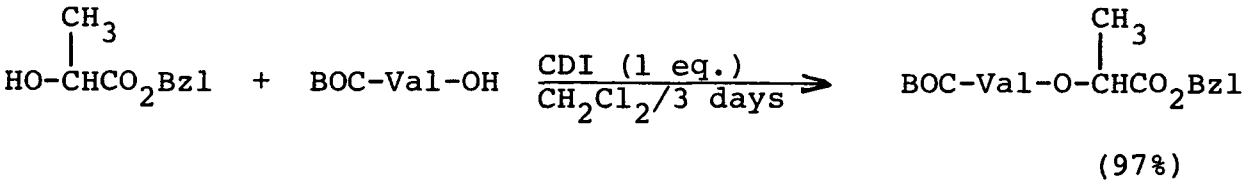


Figure 24. The Formation of a Depsipeptide Bond Using N,N'-Carbonyldiimidazole.[85]

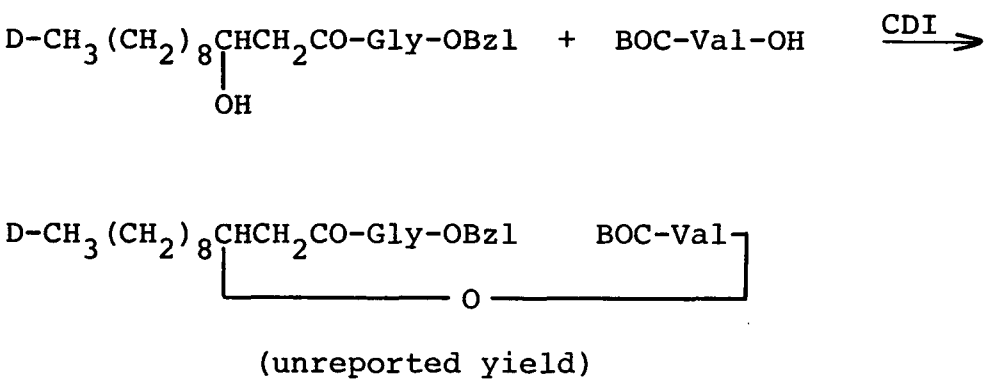
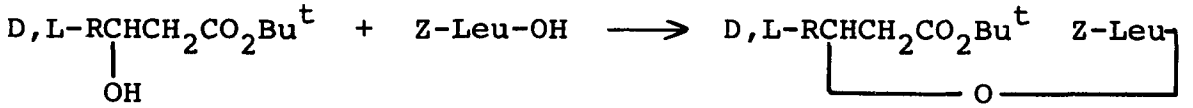


Figure 25. Depsipeptide Bond Formation Using a β-Hydroxy Acid Derivative.[14]



20 21 22

1) CDI (1 eq.) / CH_2Cl_2/3 days → no product isolated

2) CDI (1 eq.) / THF/5 days → "

3) $PhSO_2Cl$ / pyridine → "

$R = CH_3(CH_2)_{10}$-

Figure 26. The Attempted Esterifications of Benzyloxycarbonyl-L-leucine with D,L-tert-Butyl-β-Hydroxytetradecanoate.

the esterification reaction using benzenesulfonyl chloride, the desired esterified product 24 was obtained in approximately 75% yield (Figure 27). This result seems to indicate that steric hindrance due to bulk of the side chain on leucine was a factor inhibiting the previous esterification attempts.

In order to further investigate steric effects, esterifications were attempted using D,L-methyl-β-hydroxytetradecanoate (25). If the tert-butyl ester is also sterically hindering esterification then the corresponding methyl ester should provide a greater yield of depsipeptide. D,L-Methyl-β-hydroxytetradecanoate was prepared as shown in Figure 28. The use of dimethylformamide dineopentyl acetal in methanol gave the desired methyl ester (25) in 65% yield based on GLC analysis of the reaction mixture. Esterification with diazomethane[86,87] proved to be a superior method affording a 98% isolated yield of D,L-methyl-β-hydroxytetradecanoate. The results from esterifications using D,L-methyl-β-hydroxytetradecanoate are shown in Figure 29.

When benzyloxycarbonyl-L-leucine (21) was esterified with D,L-methyl-β-hydroxytetradecanoate (25) using both benzenesulfonyl chloride and N,N'-carbonyldiimidazole, crude didepsipeptide (26) was isolated in low yield (25% and 40%, respectively). The esterification of benzyloxycarbonylglycine (23) with the β-hydroxy methyl ester (25) under the same conditions gave substantially higher yields (70% and 62%, respectively). These results would seem to indicate that the steric bulk of the tert-butyl ester in compound 20 may

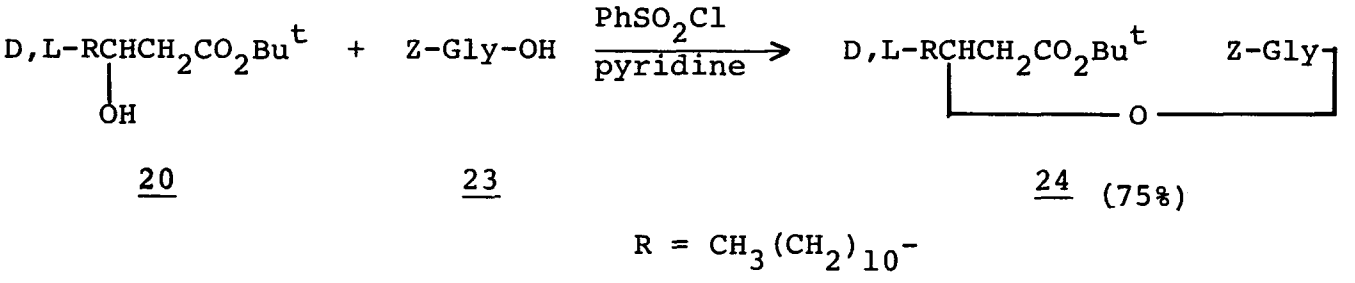


Figure 27. The Esterification of Benzyloxycarbonylglycine with D,L-tert-Butyl-β-hydroxytetradecanoate.

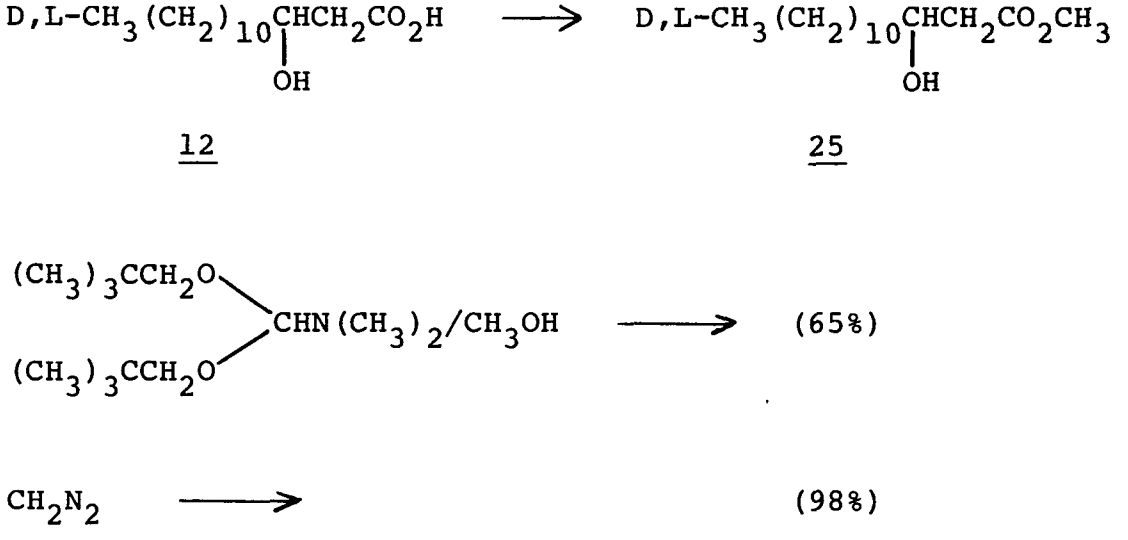


Figure 28. The Preparation of D,L-Methyl-β-hydroxytetradecanoate.

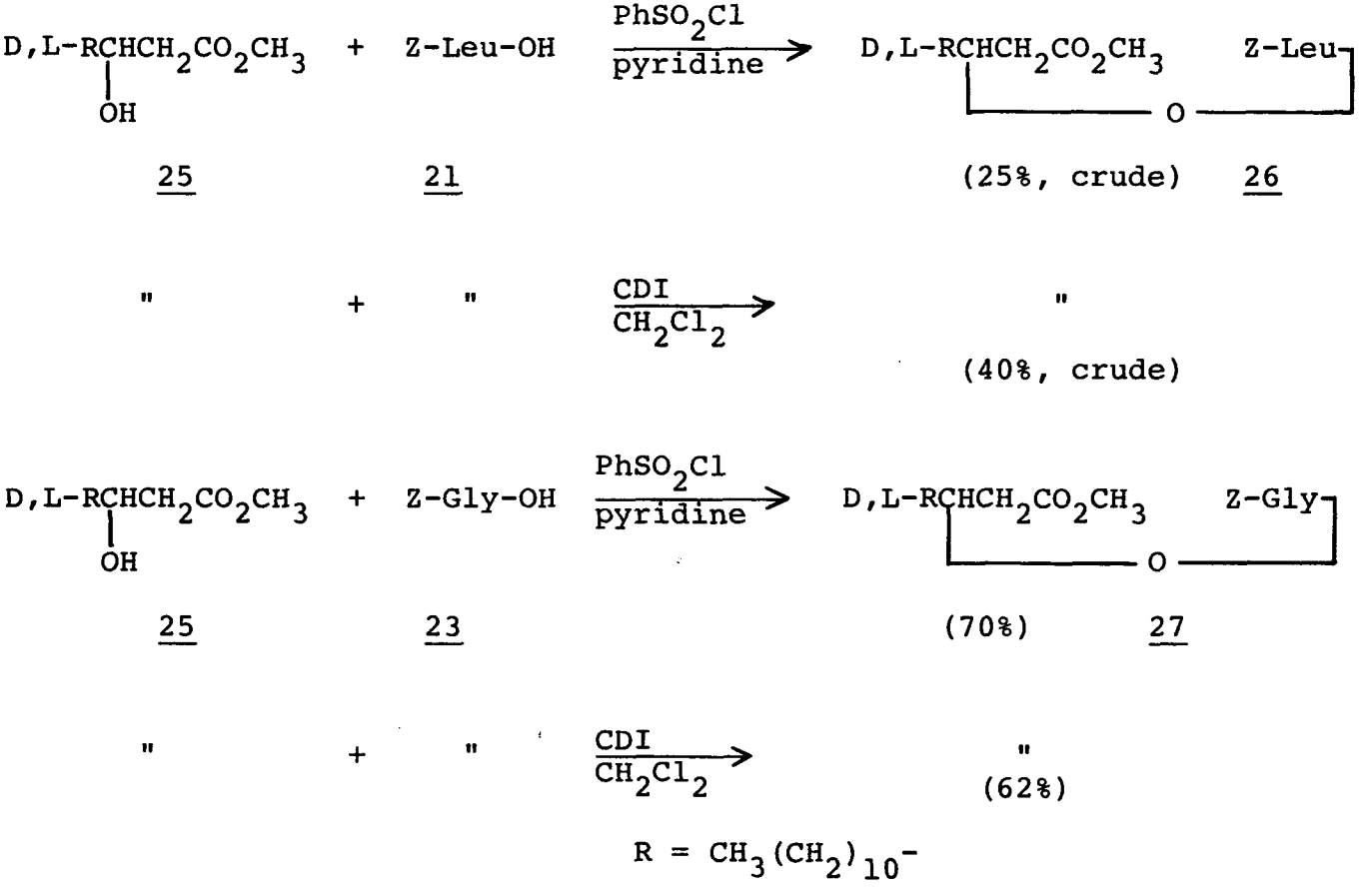


Figure 29. Esterifications Using D,L-Methyl-β-hydroxytetradecanoate.

also be a factor contributing to the failure of the attempted esterifications shown in Figure 26. In the esterification of benzyloxycarbonyl-L-leucine, a detectable yield of didepsipeptide was obtained with D,L-methyl-β-hydroxytetradecanoate (Figure 29) while no esterified product could be isolated when D,L-tert-butyl-β-hydroxytetradecanoate was used (Figure 26). However, no such increase in yield was observed for the corresponding esterifications of benzyloxycarbonylglycine. This result might be expected since glycine has no bulky side chain to interact with the tert-butyl ester. It should be noted that this is also the case with the aspartic acid derivative shown in Figure 22. There are no groups α to the β-carboxylic acid moiety of the aspartic acid derivative that is being esterified. A 69% yield of the desired product was obtained even though the sterically pretentious tert-butyl-β-hydroxytridecanoate was used.

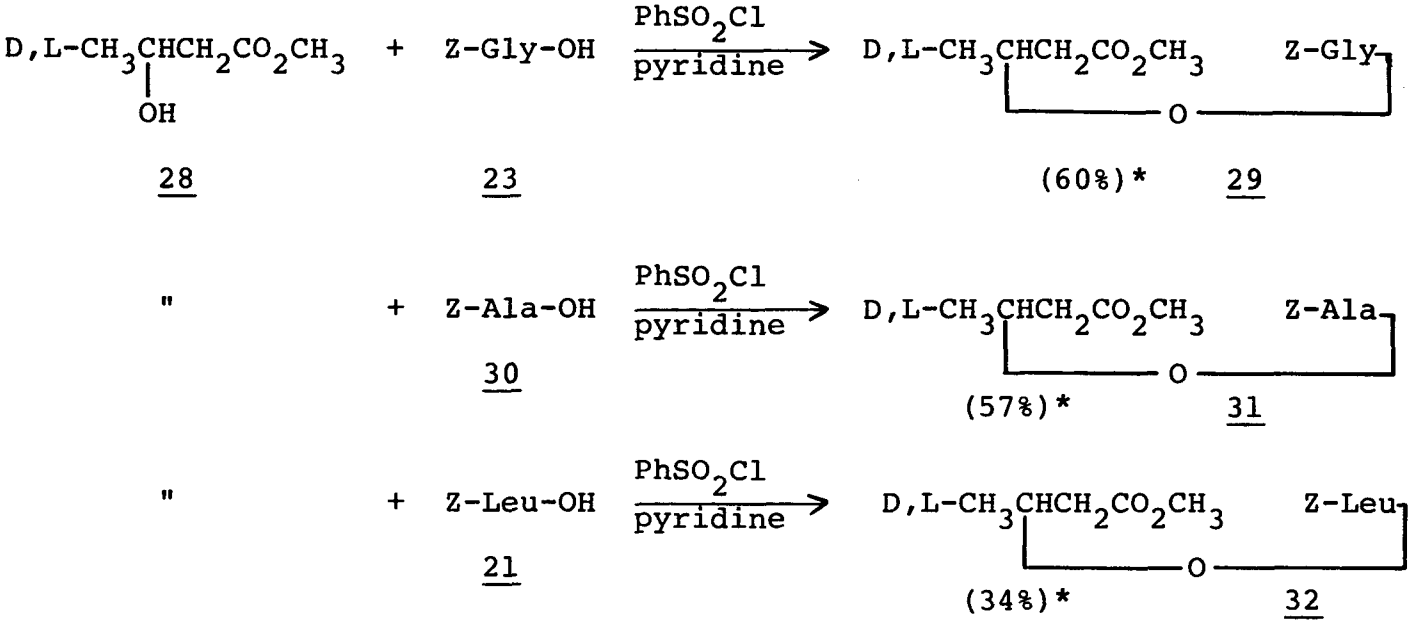
Another source of inhibition to the esterification might be the long hydrocarbon chain of β-hydroxytetradecanoic acid. In order to obtain information on the possible influences that this group might impose on the reaction, a series of esterifications were attempted using D,L-methyl-β-hydroxybutyrate (28). If the hydrocarbon chain of the β-hydroxytetradecanoic acid esters is contributing to steric hindrance in the reaction, then an increase in yield would be expected since this source of hindrance is not present in the β-hydroxybutyrate.

D,L-Methyl-β-hydroxybutyrate was prepared from D,L-β-hydroxybutyric acid and diazomethane. The results of several Z-amino acid esterifications using D,L-methyl-β-hydroxybutyrate are summarized in Figure 30.

The results in Figure 30 show that there was no increase in esterification yield when D,L-methyl-β-hydroxybutyrate, rather than D,L-methyl-β-hydroxytetradecanoate (Figure 29), was the alcohol component. This would seem to indicate that the hydrocarbon chain of the β-hydroxytetradecanoic acid esters is not a major influence. However, there was a significant decrease in yields observed when proceeding from amino acids with small or no α-side chain (benzyloxycarbonyl-L-alanine, 30, and benzyloxycarbonylglycine, 23) to an amino acid derivative with a large bulky α-side chain (benzyloxycarbonyl-L-leucine, 21). Along with the observations summarized in Figure 29, this supports the argument that the side chain steric bulk is an important factor.

In summary, it would seem that inhibitory influences in these types of esterifications result primarily from the bulk of the α-side chain of the amino acid derivative. The size of the ester group on the β-hydroxy acid also seems to exert some influence but the size of the β-side chain on the β-hydroxy acid is not a major factor.

The results of the esterification study indicate that the formation of the ester bond between benzyloxycarbonyl-L-



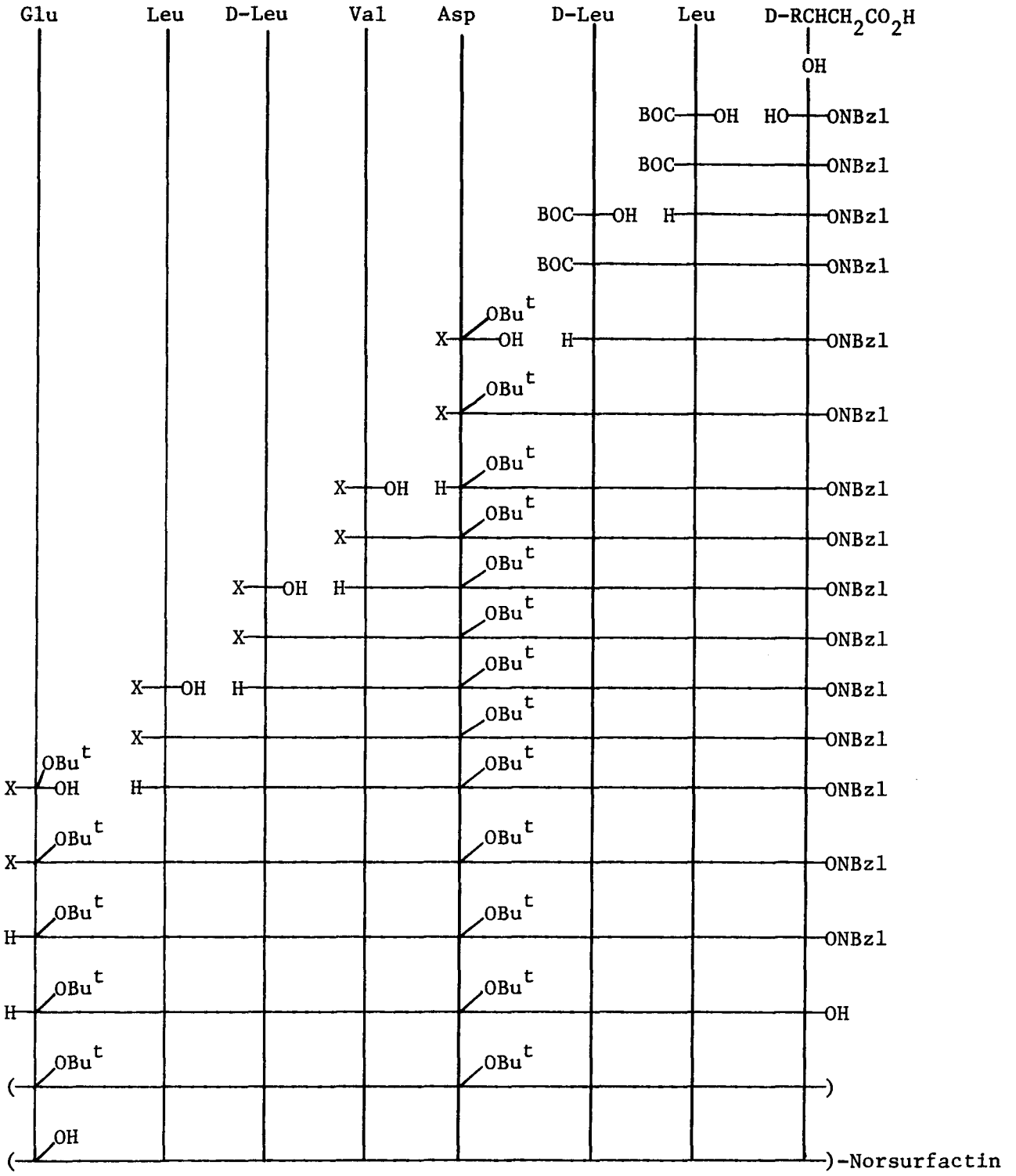
*Yields are based on GLC of crude product.

Figure 30. Esterification of Several Z-Amino Acids with D,L-Methyl-β-hydroxybutyrate.

leucine and D-tert-butyl-β-hydroxytetradecanoate in step 1 (Chart I) cannot be accomplished in acceptable yield by previously published methods.

Despite the lack of success in this initial attempt, the basic strategy of prior formation of the ester bond leading to a final cyclization through an amide bond as shown in Figure 8 still appeared to be the most desirable approach. In order to maximize the chance of success in the initial formation of the ester bond, a new synthetic strategy was developed (Chart II).

In Chart II the protecting group for the carboxylic acid moiety of β-hydroxytetradecanoic acid is a p-nitrobenzyl ester (ONBzl). The utility of this group for the C-terminal protection of amino acids during peptide synthesis was first shown by Schwarz[88] and Schwyzer.[89] It has been found that p-nitrobenzyl esters display a much greater stability under the acidic conditions used to remove benzyloxycarbonyl groups than do benzyl esters. This allows selective acidolysis of benzyloxycarbonyl groups to be accomplished in the presence of p-nitrobenzyl esters. Also, p-nitrobenzyl esters have a greater tendency to crystallize than do benzyl esters, and this became an important consideration in the synthetic strategy for norsurfactin. All of the previously prepared derivatives of β-hydroxytetradecanoic acid were either oily compounds or low melting solids that were difficult to purify. It was hoped that the introduction of a nitrobenzyl ester as the C-terminal protecting group might



X = NPS
R = $CH_3(CH_2)_{10}$-

Chart II. A Synthetic Strategy for the Stepwise Synthesis of Norsurfactin, Variation B.

impart a higher degree of crystallinity to the intermediates, hopefully making them easier to isolate and purify.

If the hydrogenolytically labile p-nitrobenzyl ester is to be used for protection of the carboxylic acid moiety of the β-hydroxy acid residue (as in Chart II) a general reversal in strategy for carboxylic acid protection (compared to the scheme outlined in Chart I) is required. The side chain carboxylic acid groups of aspartic and glutamic acids must be protected by tert-butyl esters if the previously stated criteria for carboxylic acid protection are to be complied with. A similar reversal is required for the N-terminal protecting groups on the first two leucine residues. A tert-butoxycarbonyl group (BOC-) must now be used for the N-terminal protection of these residues. The BOC group can be removed under mildly acidic conditions without disturbing the C-terminal p-nitrobenzyl ester. The use of an extremely acid sensitive N-terminal protecting group, such as the o-nitrophenylsulfenyl group, is again required when the β-tert-butylaspartate derivative is introduced, and for all subsequent couplings, so that the tert-butyl ester can be retained. Stepwise build up of the peptide would proceed in the same manner as shown in Chart I. Deprotection of the linear octadepsipeptide at both C- and N-terminal positions and a cyclization followed by removal of the tert-butyl esters on the side chains of the aspartic and glutamic acid residues should lead to norsurfactin.

A closer examination of the strategy outlined in Chart II reveals a possible problem. The literature states that o-nitrophenylsulfenyl protecting groups can be selectively removed in the presence of tert-butyl esters by exposure to very mildly acidic conditions.[78] However, the repeated exposure of the tert-butyl ester on the aspartic acid residue to such conditions required by the strategy outlined in Chart II, is cause for concern. After the incorporation of the β-tert-butyl aspartate residue into the depsipeptide, successive removal of o-nitrophenylsulfenyl groups during further stepwise elongation of the peptide chain would expose this tert-butyl ester to acidic conditions five times. This could lead to partial cleavage of the tert-butyl ester and possibly to the formation of small amounts of β-aspartyl peptides during subsequent peptide coupling steps.

In order to avoid this potential problem, an alternative fragment type synthetic strategy was developed (Chart III). The tetradepsipeptide fragment B would be prepared using the strategy summarized in Chart II. The tetrapeptide fragment A would be prepared in a stepwise fashion also. The C-terminal carboxylic acid group on valine can be protected as a methyl ester since there are no alkaline sensitive groups in the tetrapeptide. Stepwise elongation would be accomplished using a Z group for the N-terminal protection of new units during coupling; hydrogenolysis would be suitable

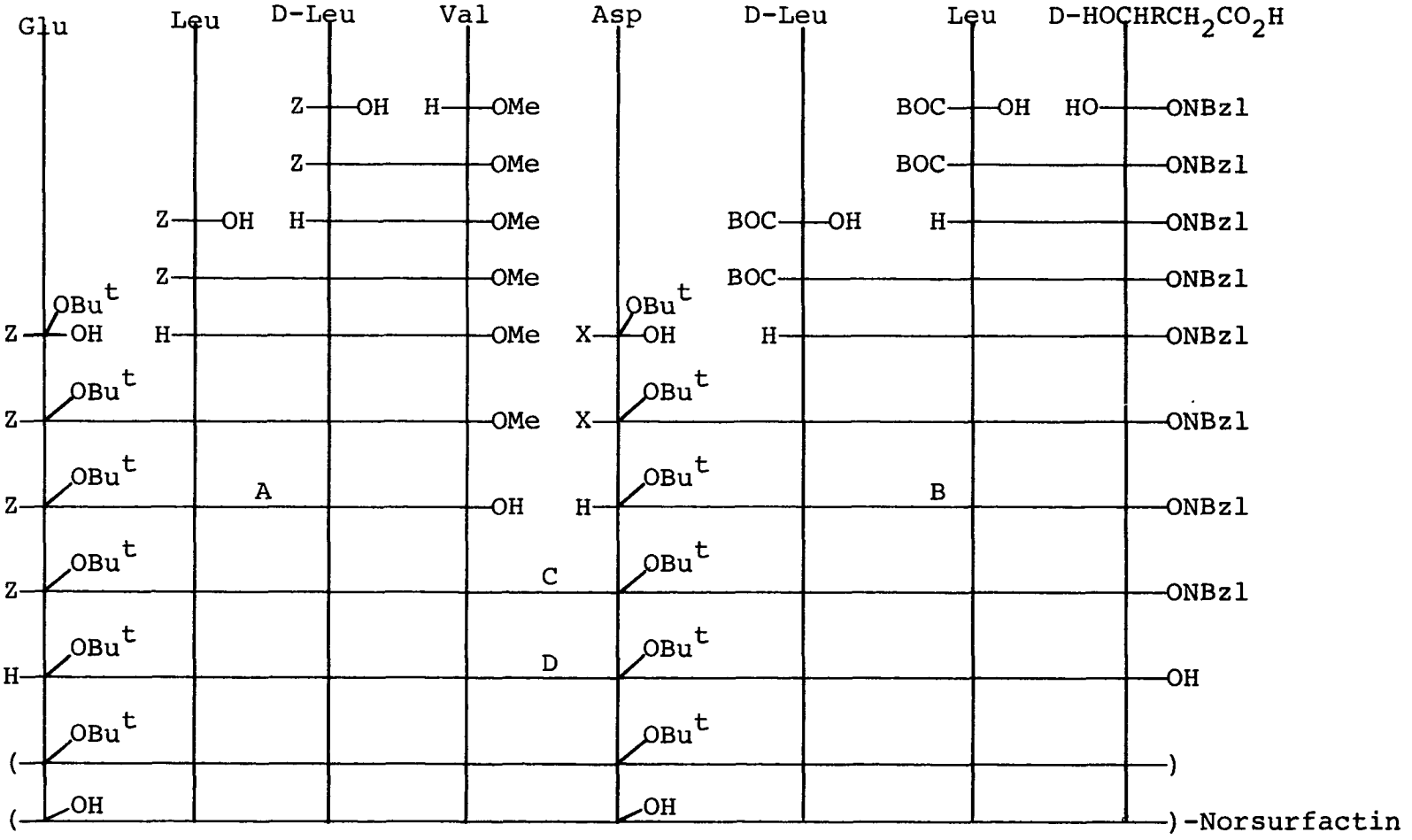


Chart III. Fragment Synthetic Strategy for Norsurfactin, Variation A.

for removal of the Z group prior to the next coupling step. The side chain γ-carboxylic acid moiety of the N-terminal glutamic acid residue would be protected by a tert-butyl ester. The C-terminal ester on the completely protected tetrapeptide would be selectively removed by saponification. The selective saponification of methyl esters in the presence of tert-butyl esters has been reported many times.[90,91] A (4+4) fragment coupling (tetrapeptide A plus tetradepsipeptide B) would lead to the completely protected octadepsipeptide C. Simultaneous removal of the N-terminal benzyloxycarbonyl group on the glutamic acid residue and the p-nitrobenzyl ester on the C-terminal β-hydroxy acid residue would give the partially protected octadepsipeptide D. Cyclization followed by acidic removal of the remaining tert-butyl ester protecting groups would lead to norsurfactin.

If this scheme were followed, then the tert-butyl ester on the β-carboxylic acid residue of aspartic acid would only be exposed to acidic conditions once, during removal of the o-nitrophenylsulfenyl protecting group on the tetradepsipeptide prior to the fragment coupling.

Following the strategy outlined in Chart III, the preparation of p-nitrobenzyl-β-hydroxytetradecanoate was accomplished using a procedure similar to that for attachment of amino acid residues to chloromethylated polystyrene solid supports in the solid phase peptide synthesis technique (Figure 31).[92]

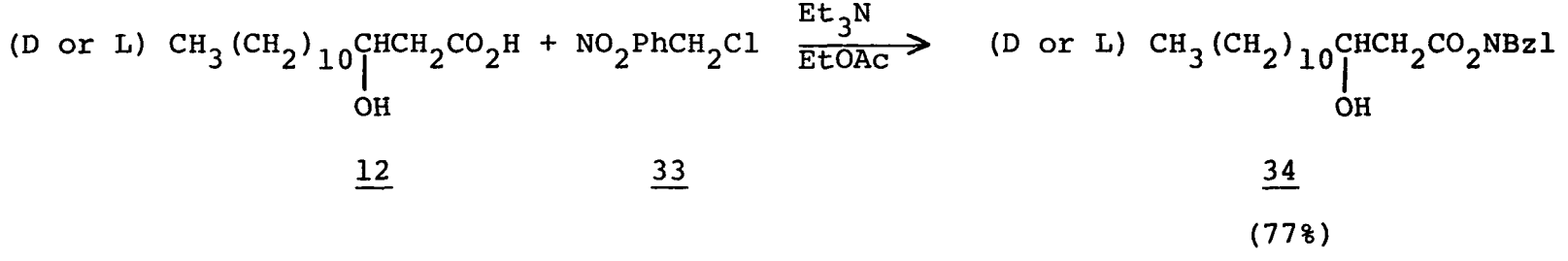


Figure 31. The Preparation of D and L p-Nitrobenzyl-β-hydroxytetradecanoate.

In separate experiments, both D and L-β-hydroxytetradecanoic acid (12) were treated with p-nitrobenzyl chloride (33) in the presence of triethylamine in ethyl acetate solvent. The desired D and L p-nitrobenzyl-β-hydroxytetradecanoates (34) were obtained as crystalline solids (m.p. 55°) in 77% yield. It was also possible to recover the unreacted optically active β-hydroxytetradecanoic acids from the reaction mixtures.

The tert-butoxycarbonyl amino acids to be used in the synthesis were prepared by the procedure of Weinstein using tert-butoxycarbonyl azide as shown in Figure 12.[93]

The problem of the formation of the ester bond between tert-butoxycarbonyl-L-leucine and D-p-nitrobenzyl-β-hydroxytetradecanoate remained to be solved. It was decided to attempt the initial reactions using tert-butoxycarbonyl-D-leucine and L-p-nitrobenzyl-β-hydroxytetradecanoate as model compounds. In this way the D-p-nitrobenzyl-β-hydroxytetradecanoate needed for the synthesis of norsurfactin could be conserved until satisfactory conditions for the esterification were developed. The enantio-intermediates obtained using the model compounds should have physical properties identical to those of the norsurfactin intermediates. Similarly the rates of reactions to form enantio-intermediates should be the same. Carrying these enantio-intermediates through the remaining steps outlined in Chart III would result in the preparation of a norsurfactin diastereomer (the configurations of the β-hydroxy acid residue and the C-terminal leucine residue being opposite to those found in norsurfactin). The physical and

biological properties of such a compound would be of added interest.

It was determined that the esterification of tert-butoxycarbonyl-D-leucine (35) with L-p-nitrobenzyl-β-hydroxy-tetradecanoate (L-34) could be satisfactorily accomplished under the conditions shown in Figure 32. The desired esterified product 36 was obtained as an oily material in 90% yield when three equivalents of both tert-butoxycarbonyl-D-leucine (35) and N,N'-carbonyldiimidazole were used and the reaction was allowed to proceed for seven days. The attainment of high yields by using a large excess of carboxylic acid substrate and N,N'-carbonyldiimidazole for the formation of depsipeptide bonds had not been reported in the literature at the time this reaction was carried out, but has since been substantiated.[16]

Esterification of tert-butoxycarbonyl-L-leucine (37) with D-p-nitrobenzyl-β-hydroxytetradecanoate (D-34) was carried out under similar conditions (Figure 33). In this case the reaction was allowed to proceed for only five days and only a 76% yield of esterified product was obtained.

Shortly after these results were obtained, a synthesis of isariin was reported in which similar esterifications were attempted (Figure 34).[15]

The low yields obtained using procedures A through C and particularly with the benzenesulfonyl chloride procedure B, substantiate our earlier failures in the attempts to esterify leucine derivatives (Figures 26 and 29). A 59%

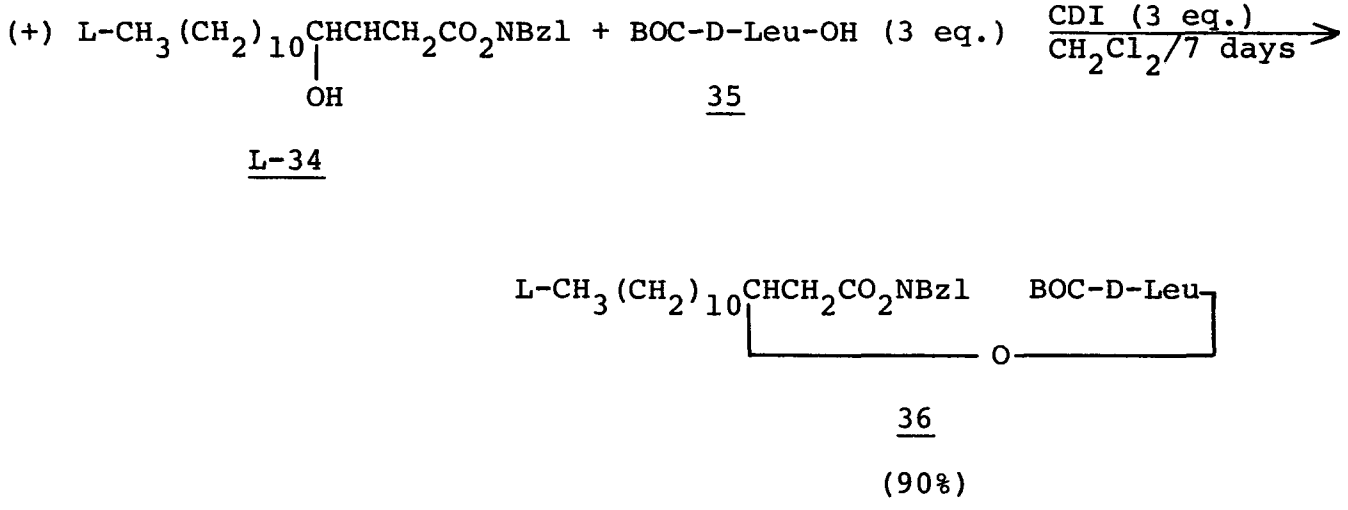


Figure 32. The Esterification of tert-Butoxycarbonyl-D-leucine with L-p-Nitrobenzyl-β-hydroxytetradecanoate.

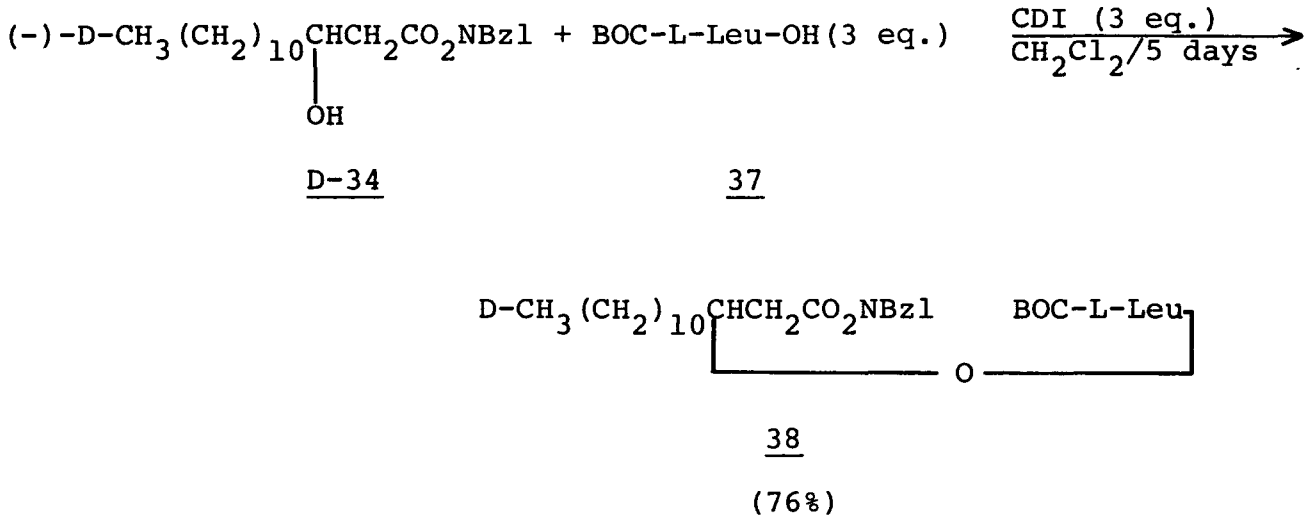


Figure 33. The Esterification of tert-Butoxycarbonyl-L-leucine.

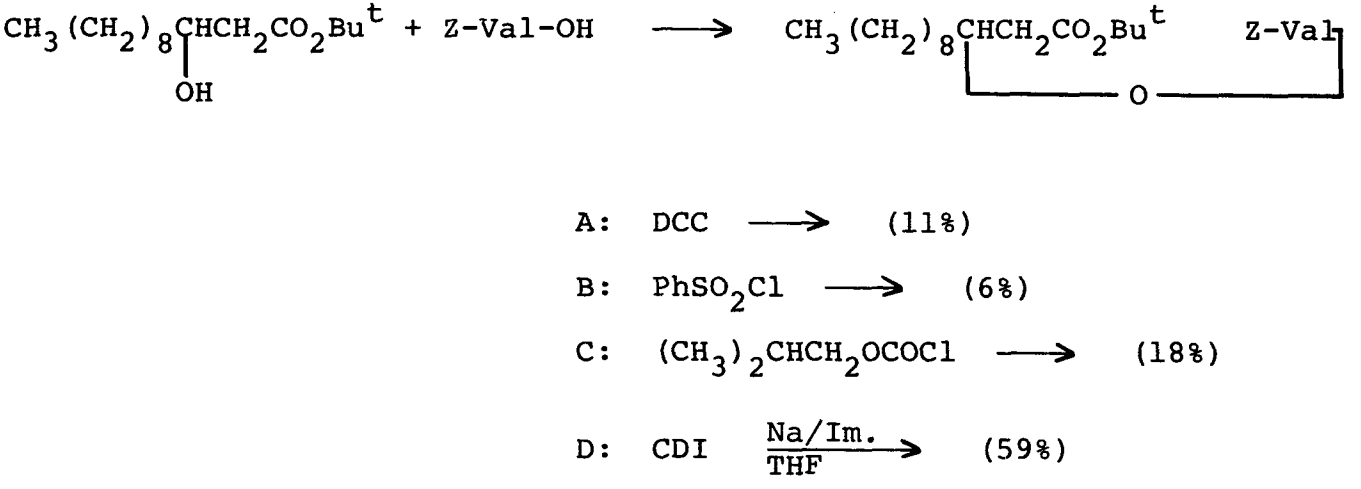


Figure 34. The Results of Esterification Reactions Attempted in Isariin Synthetic Studies.[15]

yield of esterified product was reported using one equivalent of N,N'-carbonyldiimidazole for activation in a reaction catalyzed by a small amount of a sodium-imidazole solution in tetrahydrofuran (procedure D). Our failure to obtain any esterified product in a similar system (Figure 26) using N,N'-carbonyldiimidazole alone would seem to make the catalytic procedure significant. Presumably the catalysis is a result of imidazole anion formation as shown in Figure 35.

It appears probable that the imidazole anion formed from the reaction with sodium removes a proton from the hydroxyl group of the β-hydroxy ester to form an oxygen anion. The rate of attack of this anion on the activated amino acid imidazolide is presumably faster than that of the unionized hydroxy group thus resulting in an increased yield of the esterified product.

When sodium-imidazole catalysis was used along with the conditions shown in Figure 32, a 97% yield of esterified product was obtained after allowing the reaction to proceed for only three days (Figure 36).

Continuing the synthetic scheme in Chart III, the tert-butoxycarbonyl group was cleanly removed from the L,D-didepsipeptide 36 using boron trifluoride diethyletherate in acetic acid (Figure 37).[94] The free N-terminal L,D-didepsipeptide 39 was obtained in 93% yield. The D,L-didepsipeptide 38 was similarly deprotected to give the free N-terminal D,L-didepsipeptide 40 in 92% yield.

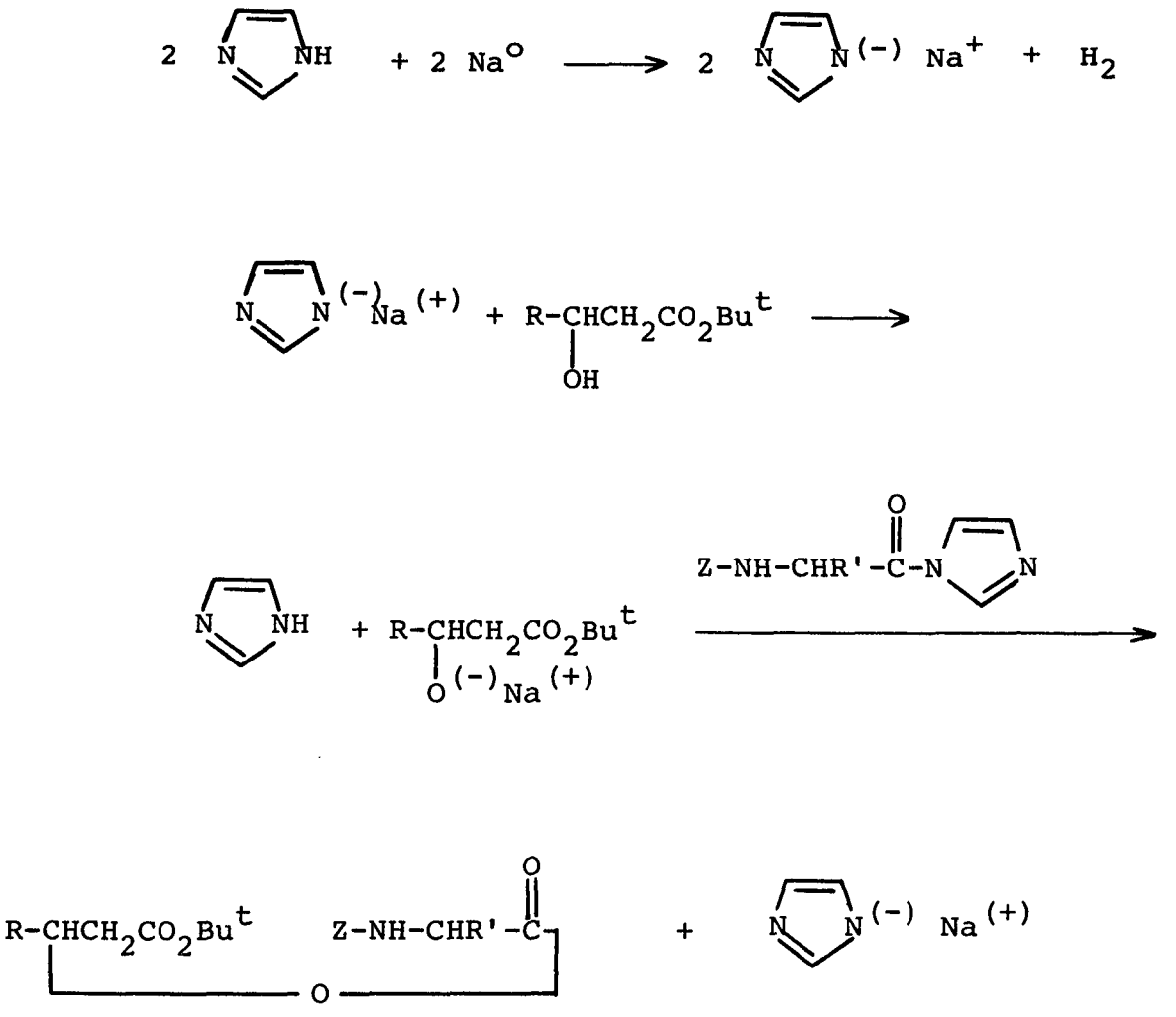
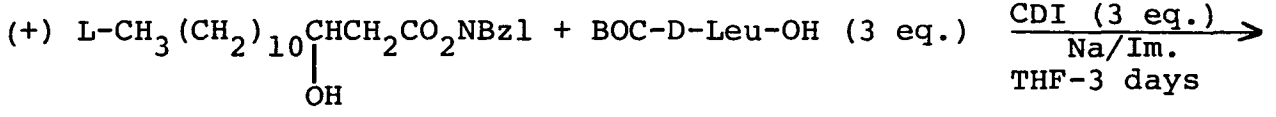
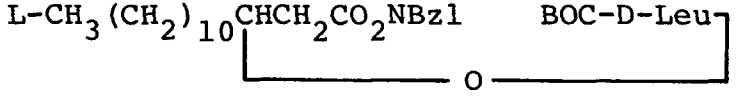


Figure 35. A Presumed Mechanism for the Sodium-imidazole Catalyzed Esterification.



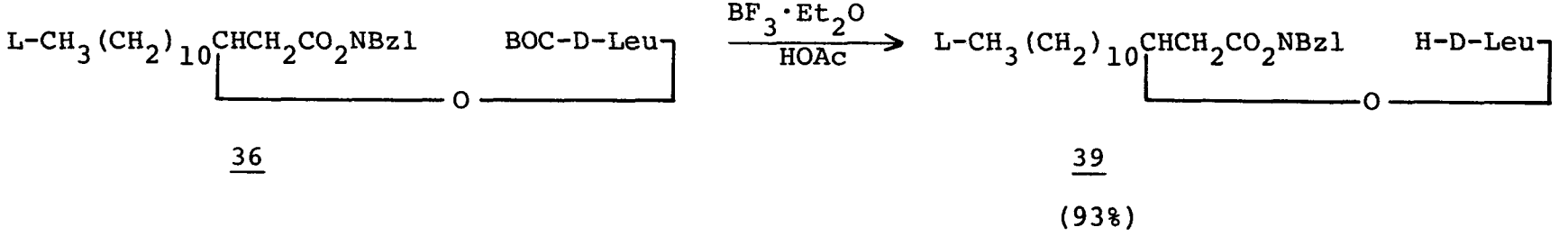
L-34 35



36

(97%)

Figure 36. The Sodium-imidazole Catalyzed Esterification of tert-Butoxycarbonyl-D-leucine.



36

39

(93%)

Figure 37. The Removal of a tert-Butoxycarbonyl Group Using Boron Trifluoride Diethyletherate.

Having formed the required ester bonds and removed the N-terminal protecting groups from the resulting didepsipeptides, the elongation of the depsipeptide fragment is the next step in the scheme shown in Chart III. This involves the formation of a peptide bond between tert-butoxycarbonyl-D-leucine and didepsipeptide 40 to form the protected tridepsipeptide 41 (Figure 38).

Numerous procedures have been developed for the formation of peptide bonds in very high yields.* The procedure that has had the greatest impact on peptide synthesis is the use of N,N'-dicyclohexylcarbodiimide (DCC) as a condensing agent. Sheehan and Hess first introduced the use of this reagent for the formation of peptide bonds in 1955.[97] Numerous other coupling reagents have since been shown to give equally good results, but N,N'-dicyclohexylcarbodiimide is still one of the most often used methods and is the primary coupling method used in Merrifield's solid phase peptide synthesis technique.[73] However, in spite of its obvious utility, N,N'-dicyclohexylcarbodiimide can also cause problems when used as a solution coupling reagent.[51] For example, the formation of hard to remove by-products and the occurrence of significant amounts of racemization on the free C-terminal activated residue during the formation of some peptides has been observed in many cases.[98] In order to avoid these

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*For reviews of coupling procedures for peptide synthesis see references 51, 95, 96.

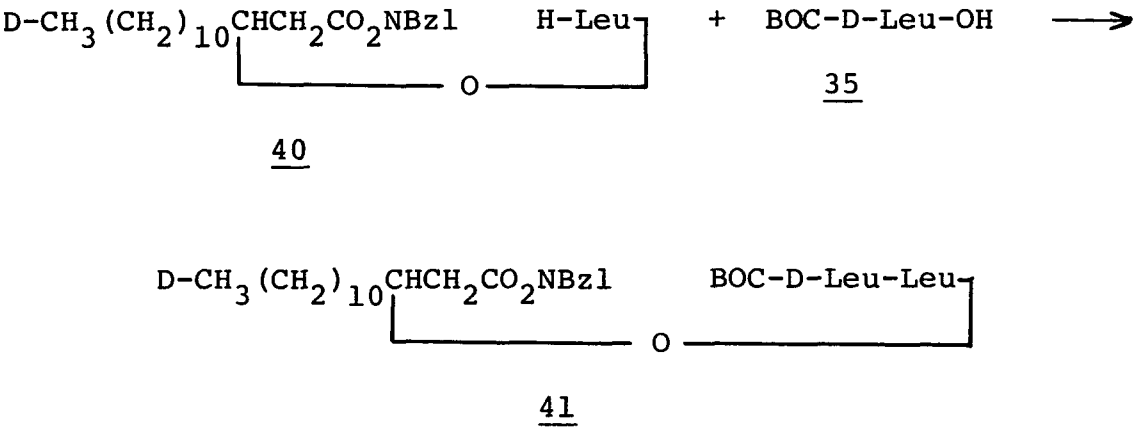


Figure 38. The Elongation of the Depsipeptide Fragment.

problems, other methods of peptide bond formation were investigated in this study.

One coupling procedure that has proven to be very successful is the N,N'-dicyclohexylcarbodiimide mediated formation of activated esters. This general procedure is outlined in Figure 39. Condensation of an appropriate activating hydroxylic reagent with an N-terminally protected amino acid using DCC yields an activated amino acid ester. Reaction of the active ester with a C-terminally protected amino acid gives a fully protected dipeptide.

The use of N-hydroxysuccinimide with DCC to form active succinimido-amino acid esters is an example of this method (Figure 40). This procedure generally gives high yields and very little racemization.[99] However, the formation of by-products resulting from a reaction between N,N'-dicyclohexylcarbodiimide and N-hydroxysuccinimide has been reported.[100]

Another active ester procedure which promotes the rapid formation of peptide bonds was introduced by Konig and Gieger.[101] Active esters formed from the reaction of N-terminally protected amino acid derivatives with 1-hydroxybenzotriazole (HOBT) and DCC (Figure 41) gave acceptable yields of peptides with very little racemization.

An independent racemization study of this method using the isotopic dilution technique[62a] indicated that the extent of racemization of the activated amino acid is less than 0.09% when the coupling is carried out in tetrahydro-

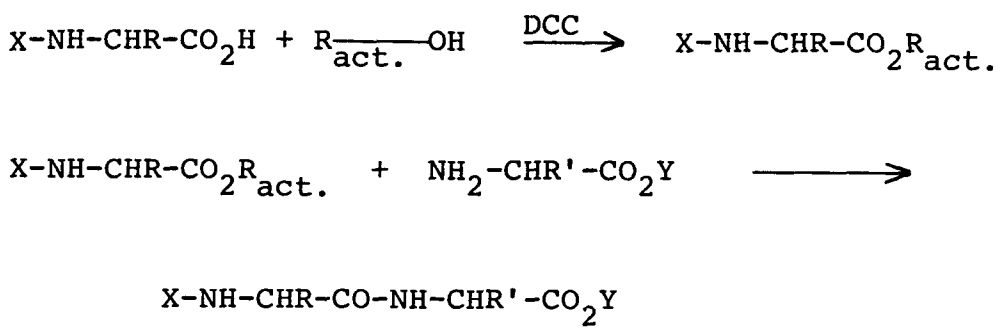


Figure 39. The Formation of Activated Esters Using N,N'-Dicyclohexylcarbodiimide.

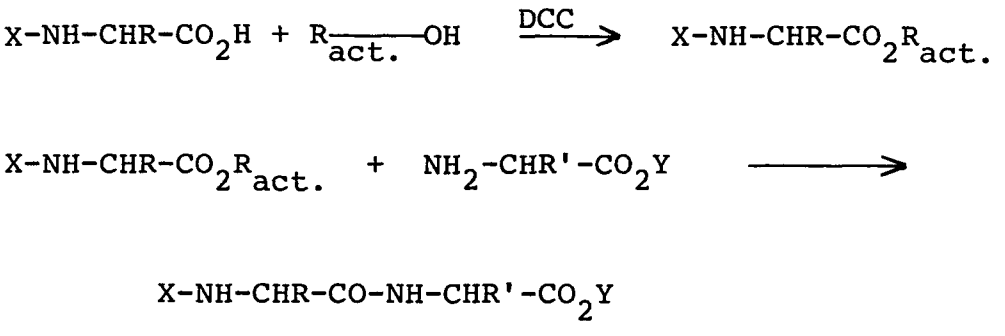


Figure 39. The Formation of Activated Esters Using N,N'-Dicyclohexylcarbodiimide.

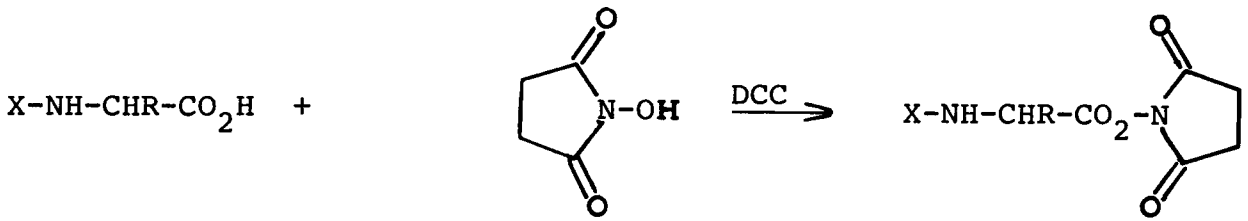


Figure 40. The Formation of N-Hydroxysuccinimide Active Esters.

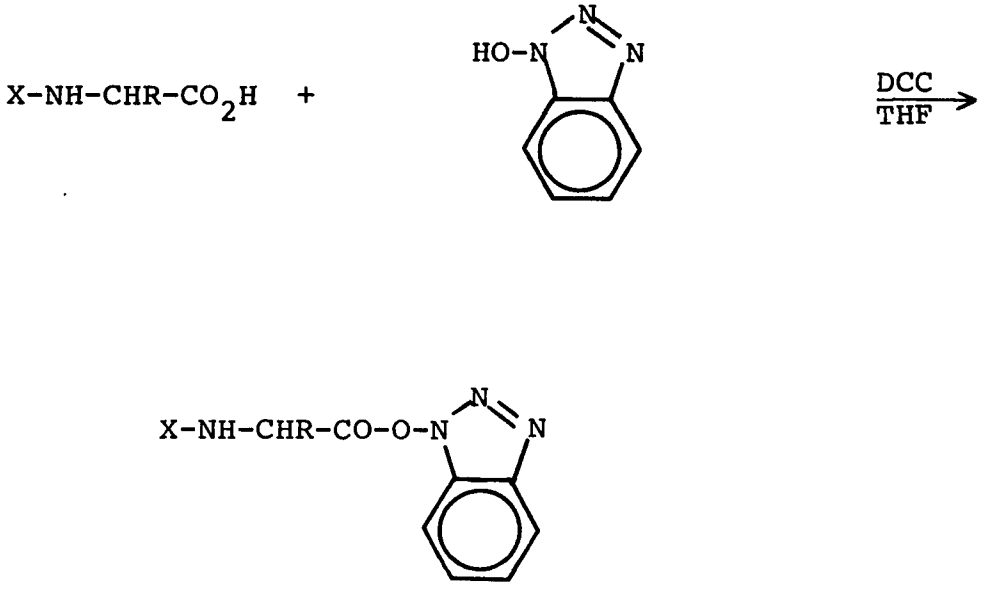
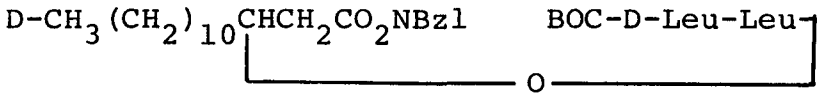


Figure 41. The Formation of 1-Hydroxybenzotriazole Active Esters.

furan.[102] An added advantage of this procedure is that the active ester intermediate need not be isolated prior to reaction with the C-terminally protected residue. Since it is also a clean and rapid procedure, the DCC-HOBT method appeared to be very attractive for use in the norsurfactin synthetic sequence.

It was decided to carry the enantiomeric model compound concept one step further in order to investigate the formation of peptide bonds with depsipeptide intermediates. The preparation of the L,D,L-tridepsipeptide 42 was accomplished in 98% yield using the DCC-HOBT procedure (Figure 42). Similarly, the protected D,L,D-tridepsipeptide 43 was obtained in 98% yield using the same procedure.



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The tert-butoxycarbonyl protecting groups on compounds 42 and 43 were removed using boron trifluoride diethyletherate in acetic acid to give the free N-terminal tridepsipeptides 44 and 45 in 93% and 94% yields, respectively. Tridepsipeptide 44 was then used as a model compound in exploratory studies on the preparation of the tetradepsipeptide fragment B in Chart III.

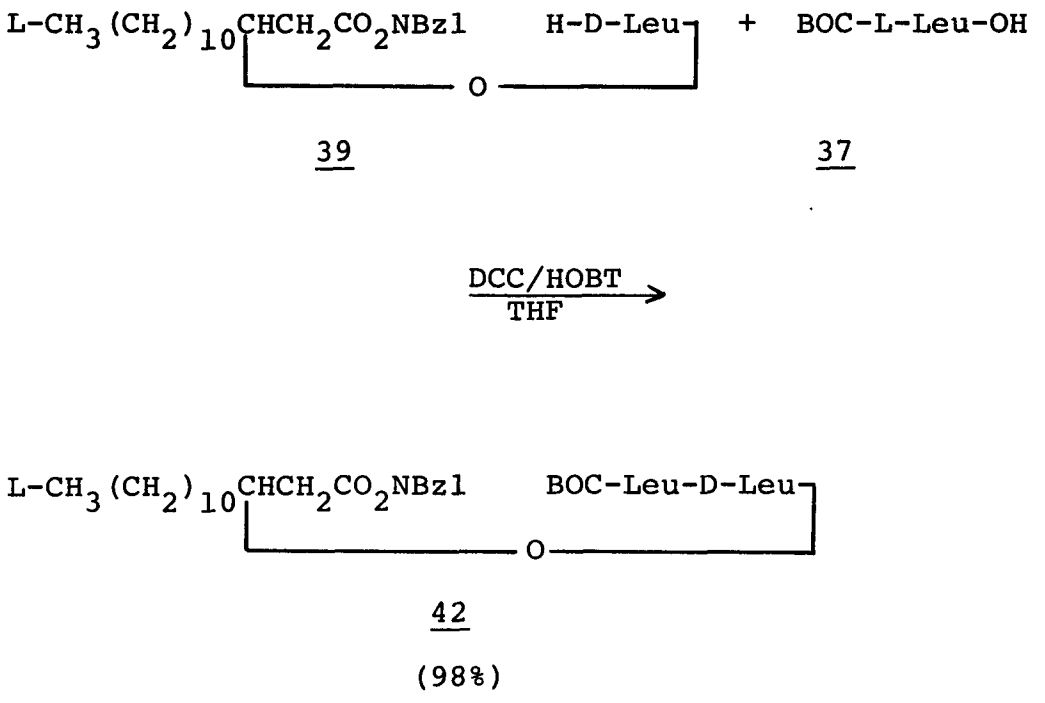
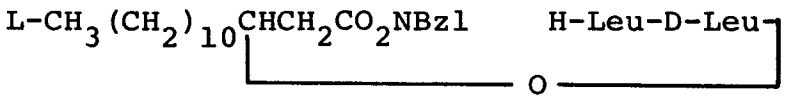


Figure 42. The Formation of the L,D,L-Tridepsipeptide.



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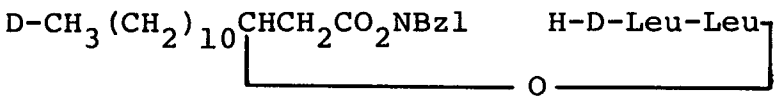

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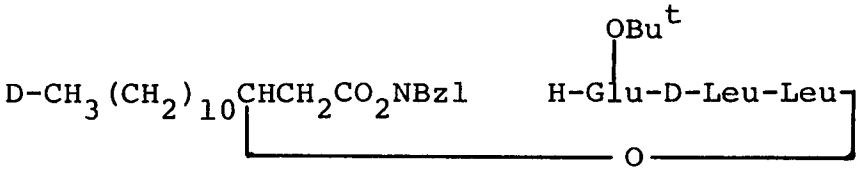


Fragment B

The preparation of the suitably protected aspartic acid residue needed for the elongation of the depsipeptide fragment was accomplished by the series of reactions shown in Figure 43. Benzyloxycarbonyl-(β-tert-butyl)-aspartate (46) was prepared by the method of Schwyzer.[103] The benzyloxycarbonyl group was then removed by catalytic hydrogenation to give β-tert-butylaspartate (47) in 84% yield. Reaction of this derivative with o-nitrophenylsulfenyl chloride (48) in aqueous sodium hydroxide solution followed by treatment with dicyclohexylamine (DCHA) gave the o-nitrophenylsulfenyl-(β-tert-butyl)-aspartate α-dicyclohexylamine salt (49) in 60% yield. o-Nitrophenylsulfenyl-(β-tert-butyl)-aspartate (50) was obtained quantitatively upon treatment of salt 49 with 10% aqueous citric acid.

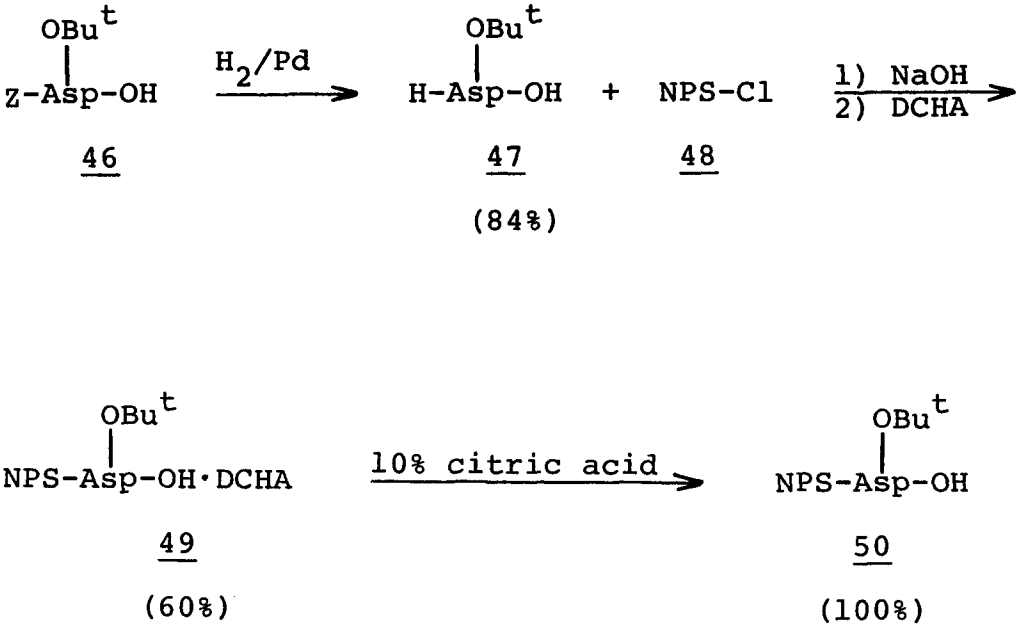


Figure 43. The Preparation of o-Nitrophenylsulfenyl-(β-tert-butyl)-aspartate (50).

The model tetradepsipeptide 51 was then prepared from compound 50 and tridepsipeptide 44 in 70% yield using the 1-hydroxybenzotriazole procedure (Figure 44).

Having prepared tetradepsipeptide fragment 51, a diastereomeric model for fragment B in Chart III, attention was turned to the preparation of the tetrapeptide fragment A needed to complete the synthetic strategy summarized in Chart III.

Benzyloxycarbonyl-(β-tert-butyl)-L-glutamyl-L-leucyl-D-leucyl-L-valine methyl ester (59) was prepared as outlined in Figure 45.

The protected dipeptide 54 was prepared in 82% yield from L-valine methyl ester hydrochloride (53)[104] and benzyloxycarbonyl-D-leucine (52) by the DCC-HOBT procedure using an equivalent of N-methyl morpholine (NMM) to free the amino group of valine from the hydrochloride salt. The benzyloxycarbonyl group was removed hydrogenolytically and the resulting free N-terminal dipeptide 55 was coupled with benzyloxycarbonyl-L-leucine (21) to yield the protected tripeptide 56 in 55% yield (from dipeptide 54). The free N-terminal tripeptide 57 was obtained in 86% yield by hydrogenolytic removal of the benzyloxycarbonyl group from 56. The completely protected tetrapeptide 59 was prepared from tripeptide 57 and benzyloxycarbonyl-(γ-tert-butyl)-glutamate[105] (58) in 86% yield using the DCC-HOBT procedure.

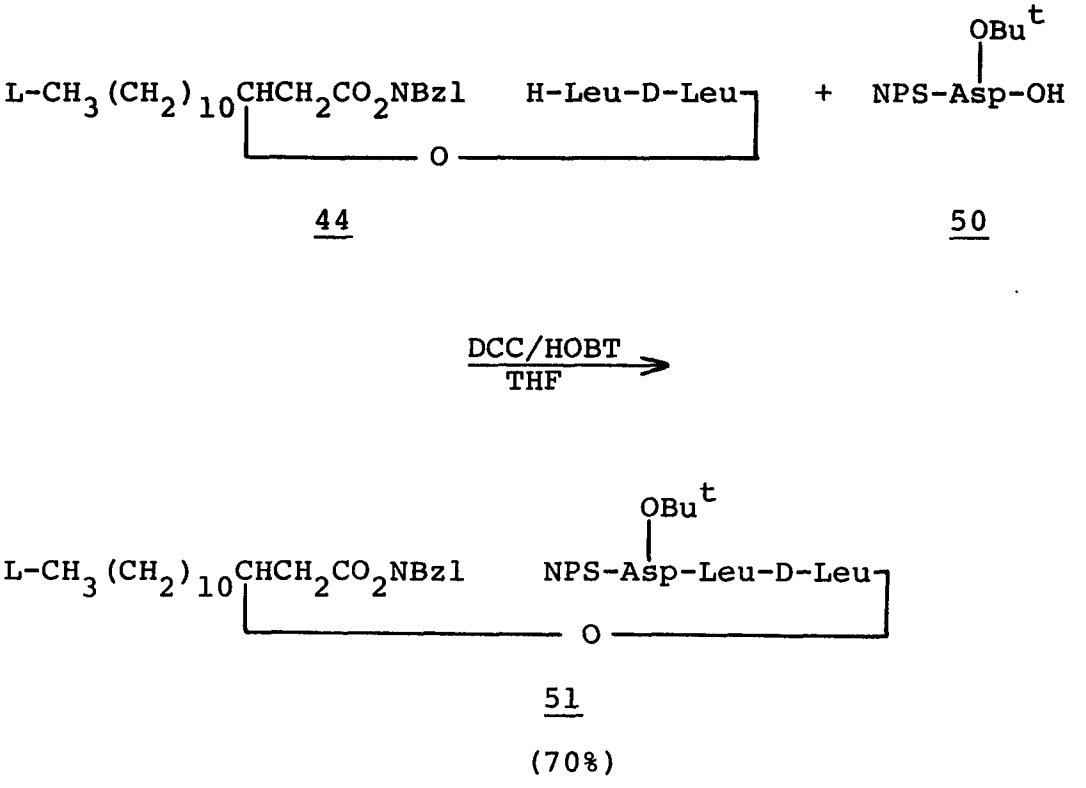


Figure 44. The Preparation of Tetradepsipeptide 51.

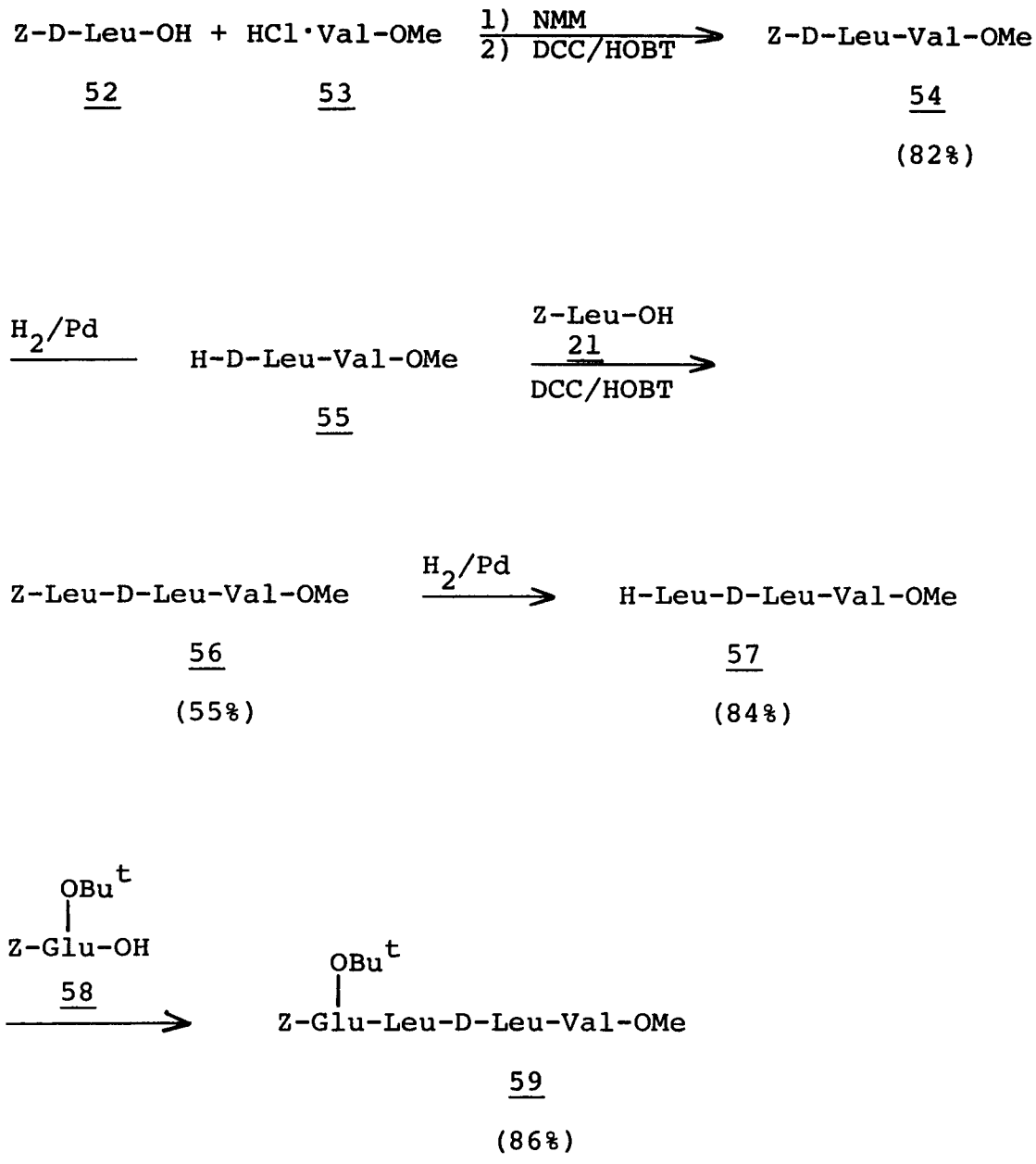


Figure 45. The Preparation of the Protected Tetrapeptide Fragment 59.

With the preparation of both a model tetradepsipeptide 51 and tetrapeptide 59 having been accomplished, only the selective removal of the N- and C-terminal protecting groups remained to be achieved before a fragment coupling could be attempted. The o-nitrophenylsulfenylgroup had to be removed from tetradepsipeptide 51 and the methyl ester had to be removed from tetrapeptide 59 in order to obtain the required intermediates.

The selective removal of o-nitrophenylsulfenyl groups from depsipeptides also containing tert-butyl esters has been reported by Ovchinnikov (Figure) 46.[41] In both examples a and b in Figure 46, the o-nitrophenylsulfenyl group was removed from the depsipeptide, cleanly and in high yield, by brief treatment with two equivalents of hydrogen chloride as a 0.3N solution in chloroform. The solid depsipeptide hydrochlorides were obtained in good purity by triturating the residue obtained by concentration of the reaction mixtures with petroleum ether.

This procedure was followed using model tetradepsipeptide 51 (Figure 47). Several attempts to selectively cleave the o-nitrophenylsulfenyl group led only to complex mixtures of oily products. Thin layer chromatographic analysis of the mixtures showed ninhydrin positive compounds as well as starting protected tetradepsipeptide indicating that some cleavage of the o-nitrophenylsulfenyl group had occurred. However, recovery of the desired tetradepsipeptide hydrochloride (60·HCl) from these oily mixtures could not

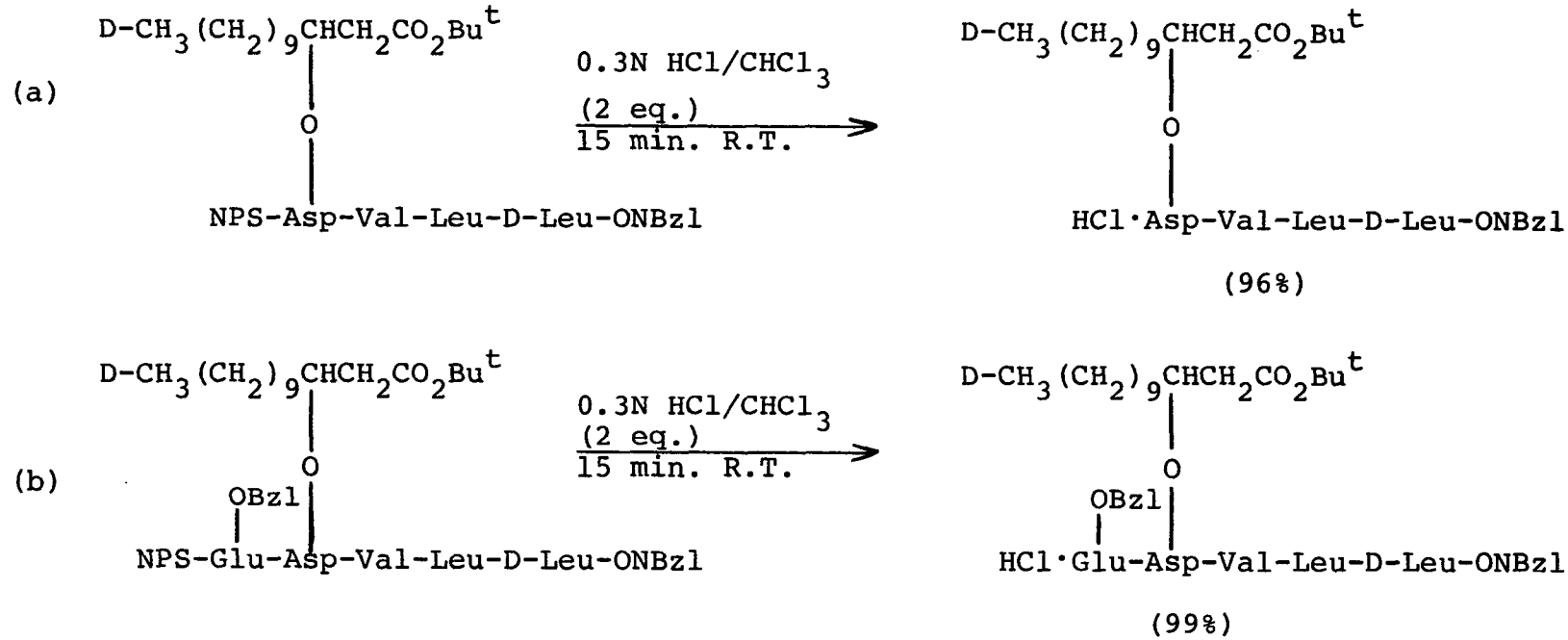


Figure 46. The Selective Removal of o-Nitrophenylsulfenyl Protecting Groups in the Presence of tert-Butyl Esters.

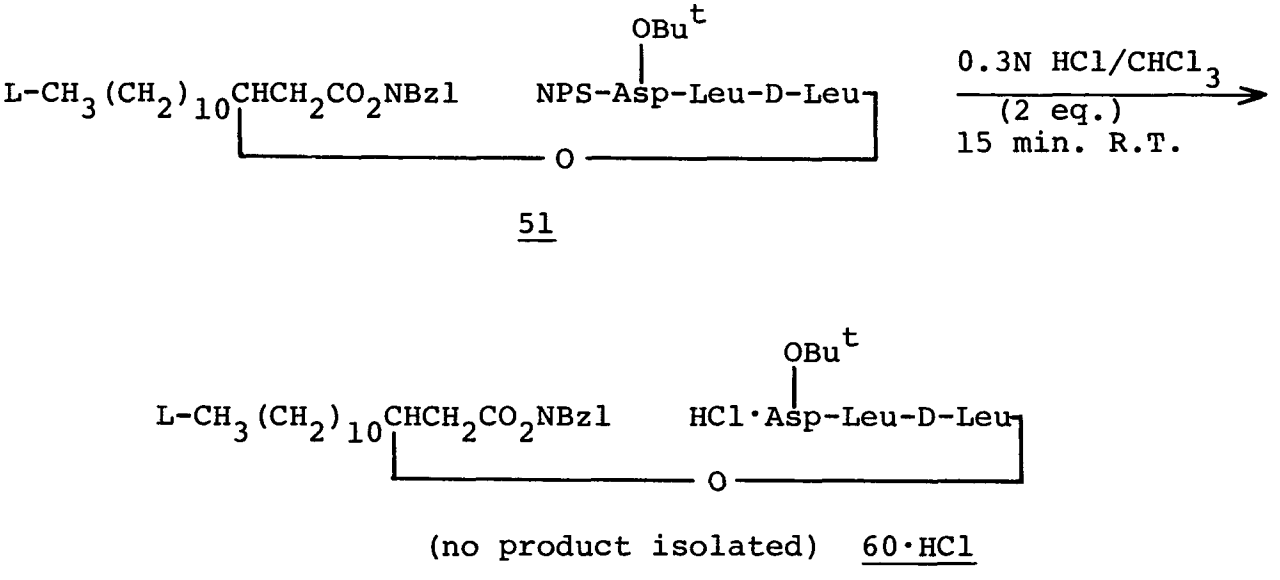


Figure 47. Attempted Selective Removal of the o-Nitrophenylsulfenyl Group Using HCl in Chloroform.

be accomplished

Several alternative o-nitrophenylsulfenyl removal techniques were also attempted using model tetradepsipeptide 51 (Figure 48).

The use of ammonium thiocynate with 2-methyl indole (procedure A) under the conditions described by Wünsch[106] resulted in very little detectable cleavage of the o-nitrophenylsulfenyl group from the tetrapeptide. Treatment with thiophenol in dimethylformamide[79] (procedure B) gave similar results. Attempted cleavage of the o-nitrophenylsulfenyl group using 88% formic acid (procedure C), under the conditions reported by Kinoshita[74] for the selective removal of N-terminal tert-butoxycarbonyl groups from peptides containing tert-butyl esters, again resulted in a complex oily mixture and none of the desired free N-terminal tetradepsipeptide could be obtained.

At this juncture it appeared that an efficient procedure for the selective removal of the o-nitrophenylsulfenyl group from tetradepsipeptide 51 could not be achieved. Therefore, the completion of the fragment type synthetic scheme outlined in Chart III was not possible.

In order to continue with the synthesis and also utilize the previously prepared synthetic intermediates, a modified fragment type synthetic scheme was developed (Chart IV).

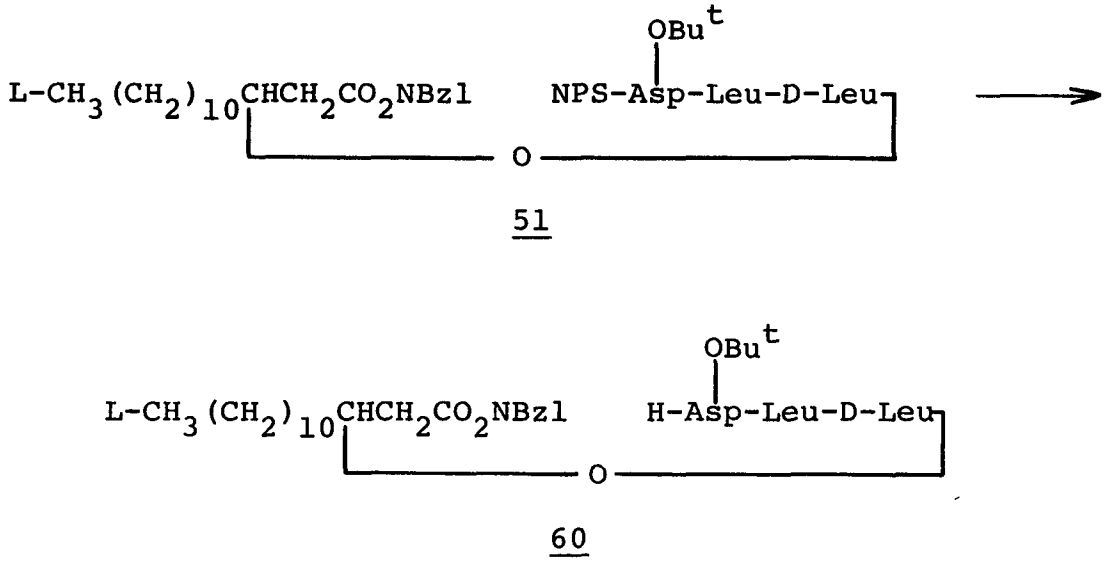
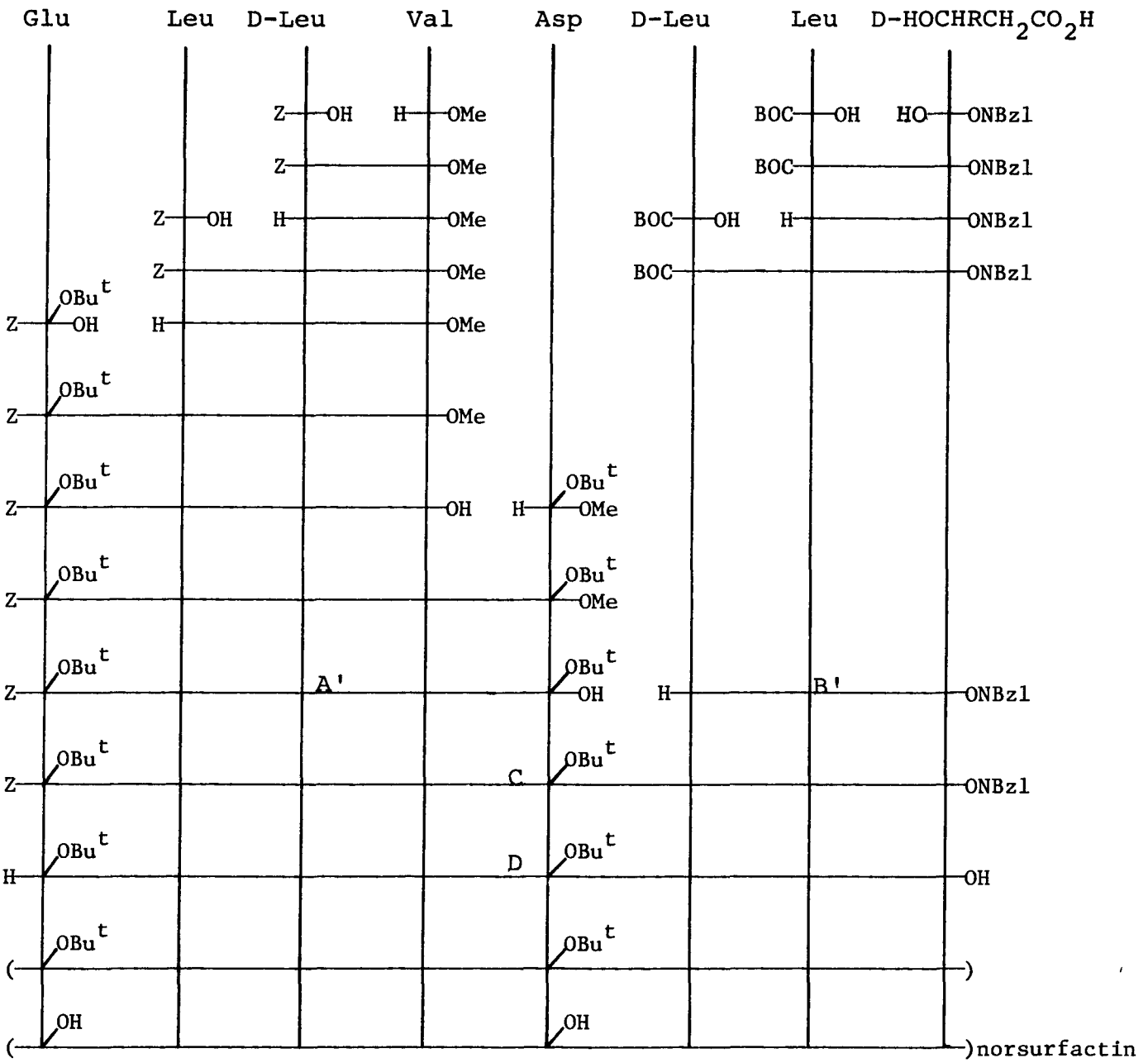


Figure 48. Additional Attempts to Remove the o-Nitrophenylsulfenyl Group.



R = $CH_3(CH_2)_{10}$-

Chart IV. Fragment Synthesis of Norsurfactin, Variation B.

This route is similar to the scheme outlined in Chart III, however, instead of a (4+4) fragment coupling step, a (5+3) fragment coupling is proposed.

The Chart IV scheme does not require the use of acid sensitive N-terminal protecting groups such as the o-nitrophenylsulfenyl group. By incorporating both side chain tert-butyl ester protecting groups into the five residue peptide fragment A', the tridepsipeptide fragment B' remains free of acid sensitive esters. The N-terminal leucine residue on the tridepsipeptide is protected by a tert-butoxycarbonyl group which can be removed with mild acid leaving the C-terminal p-nitrobenzyl ester intact. Both the tridepsipeptide fragment B' (compound 45) and its enantiomer (compound 44) had been prepared previously during this project.

Protection of the pentapeptide fragment in Chart IV is analogous to protection of the tetrapeptide fragment in Chart III. The α-carboxylic group of the aspartic acid residue can be protected as a methyl ester. This ester should then be removable by selective saponification without disturbing either of the tert-butyl ester groups to afford the pentapeptide fragment A'.* A (5+3) fragment coupling

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*It has been shown that β-tert-butyl esters on aspartic acid residues incorporated in peptides are, in some cases, base labile due to participation of the C-terminal amide nitrogen in ester hydrolysis.[107,108] This unusual sensitivity to base should not be encountered in this case since the aspartic acid residue is the C-terminal residue of the peptide and there is no C-terminal amide nitrogen to assist saponification of the tert-butyl ester.

would lead to the same protected octadepsipeptide derivative (C) proposed in Chart III. The remaining steps leading to norsurfactin are identical in both the Chart III and Chart IV schemes.

The preparation of peptide fragment A' would normally proceed in a stepwise fashion from the α-methyl-β-tert-butyl aspartate residue (in a manner similar to the preparation of the tetrapeptide fragment A in Chart III) followed by selective saponification of the C-terminal methyl ester. However, it was decided to attempt the preparation of the pentapeptide A' using the previously prepared tetrapeptide 59, as shown in Figure 49. The methyl ester was removed from tetrapeptide 59 using 3.1 equivalents of sodium hydroxide in a 75% aqueous dioxane solution. The free C-terminal tetrapeptide 60 was obtained in 71% yield. Tetrapeptide 60 was then coupled with α-methyl-β-tert-butyl aspartate hydrochloride (61)[109] using the DCC-HOBT technique, with prior neutralization of the hydrochloride salt by addition of N-methylmorpholine (NMM). The desired protected pentapeptide 62 was obtained in 65% yield. The C-terminal methyl ester was selectively saponified using 3.1 equivalents of sodium hydroxide in 75% aqueous dioxane to afford the desired pentapeptide fragment 64 in 82% yield.

Having successfully prepared the desired deprotected peptide and depsipeptide fragments, the (5+3) fragment couplings were attempted. The DCC-HOBT technique was again chosen to mediate peptide bond formation and the time of

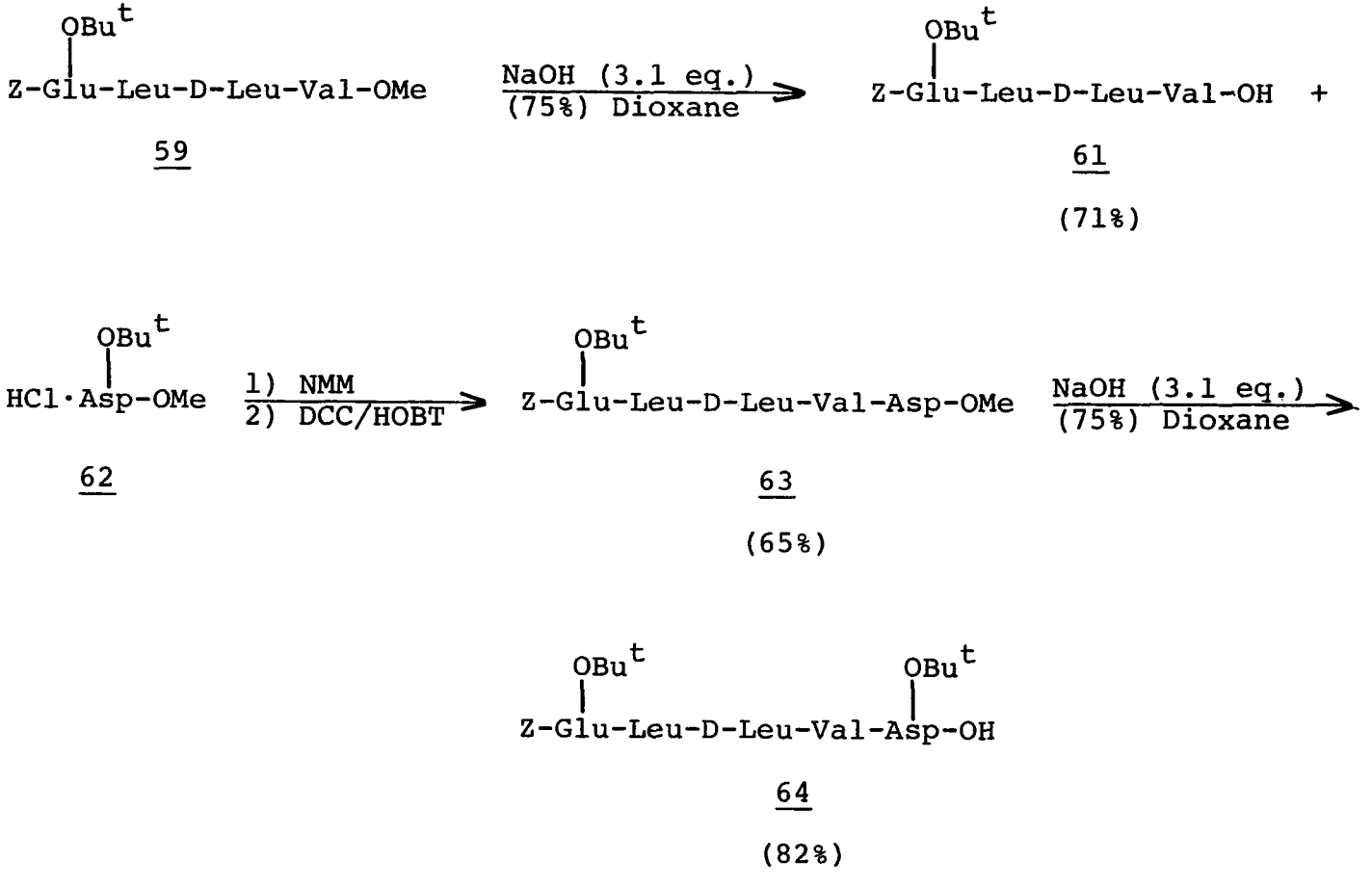


Figure 49. The Preparation of Pentapeptide A'.

reaction was extended to five days. Using this method, the fragment coupling between pentapeptide 64 and model tridepsipeptide 44 gave the protected octadepsipeptide 65 in 60% yield (Figure 50).

When this procedure was repeated using pentapeptide 64 and tridepsipeptide 45, compound 66, the acyclic octadepsipeptide precursor to norsurfactin, was obtained in 75% yield (Figure 51).

Removal of the benzyloxycarbonyl group from the N-terminal glutamic acid residue and the p-nitrobenzyl ester from the C-terminal D-β-hydroxytetradecanoic acid residue of octadepsipeptide 66 was accomplished by catalytic hydrogenation (Figure 52). The N- and C-terminally deprotected octadepsipeptide 67 was obtained in 94% yield.

The next step in the synthetic scheme (Chart IV) is the cyclization of derivative 67. The tendency of a linear peptide to cyclize is generally dependent upon the number and the configuration of the residues in the peptide. The conditions required for the successful cyclization of peptide derivatives via the formation of peptide bonds are not the same as those required for normal peptide bond formation.[1] Best results have been obtained when the carboxyl activation and cyclization steps are separable. Cyclization, like normal peptide bond formation, requires an activated carboxyl group and a free amino group. Activation of the carboxyl group being a bimolecular process is generally performed under conditions of high concentrations of reagents in order to

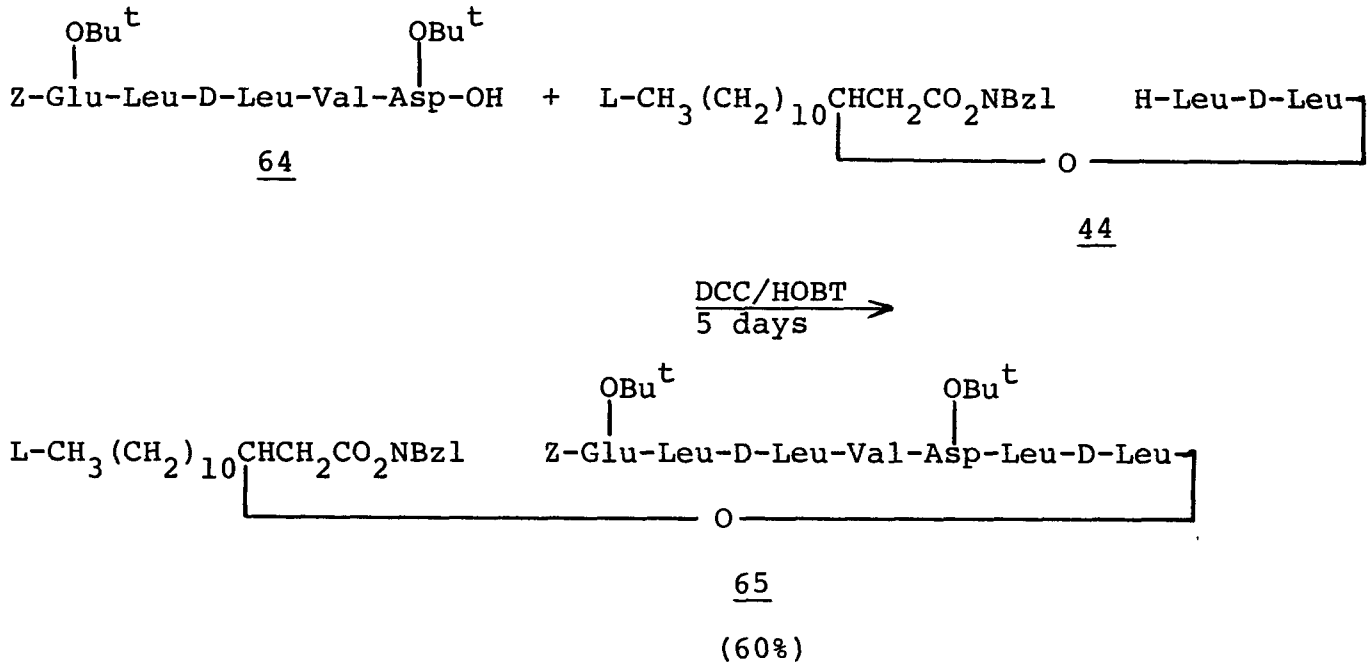


Figure 50. The Preparation of Model Octadepsipeptide 65.

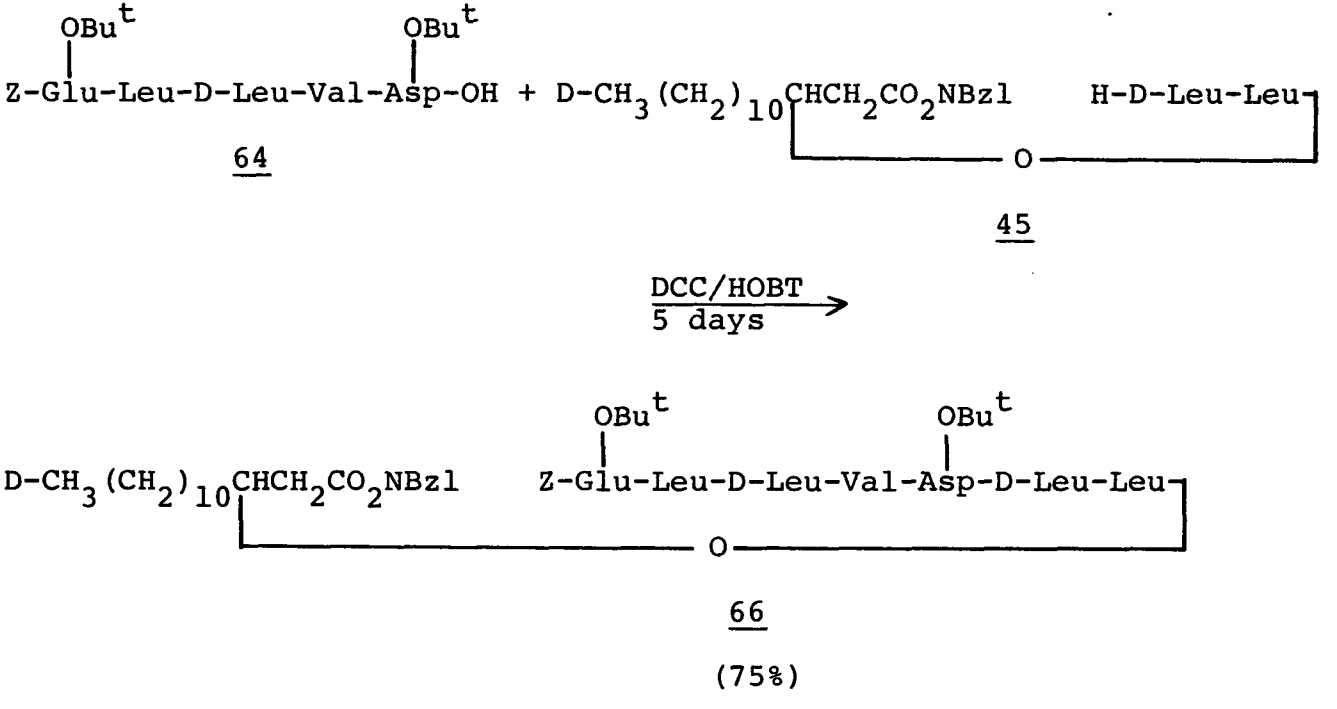


Figure 51. The Preparation of the Octadepsipeptide Precursor to Norsurfactin

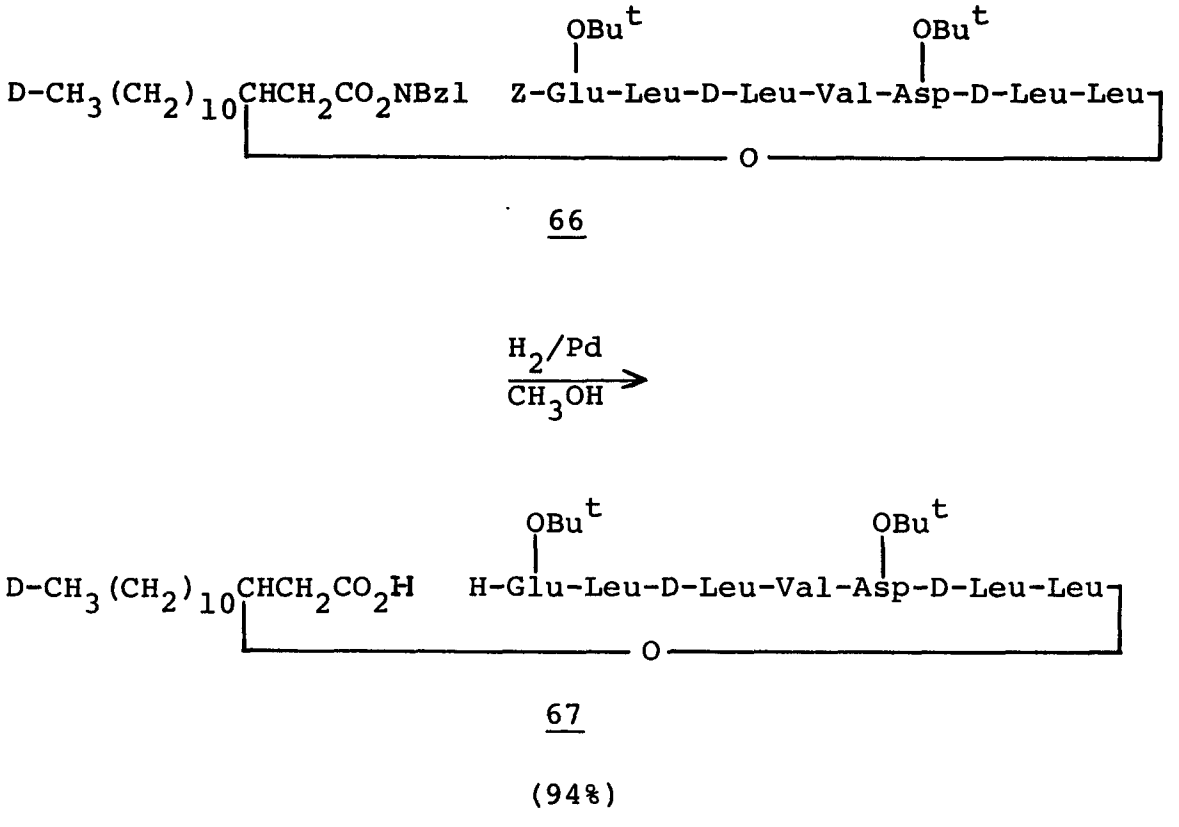
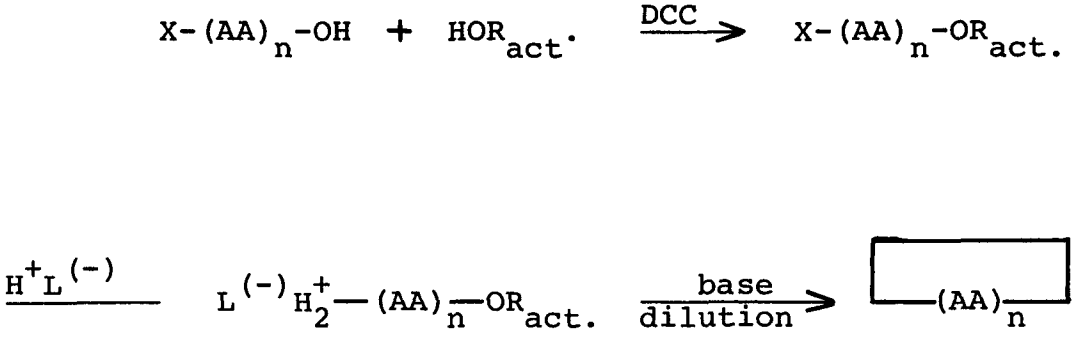


Figure 52. The Hydrogenolytic Removal of the C- and N-Terminal Protecting Groups from Octadepsipeptide 66.

maximize the formation of the activated species. If the N-terminal amino group of the peptide is left unprotected under such conditions, intermolecular reactions take place leading to polymeric products, and the yield of intramolecular cyclization product is very low. If, however, the activation and cyclization steps are separable then the carboxyl group can be activated under conditions of high concentrations of reagents while the amino group is protected to prevent polymerization. Cyclization may then be accomplished by generating the free amino group and allowing intramolecular substitution to take place under dilute conditions (10^{-3}-10^{-4} M). In this manner the yield of cyclic product is maximized and the formation of oligomers and polymers is minimized. Activated esters have been found to give good results in cyclization sequences (Figure 53).

This type of cyclization cannot be applied to the synthetic strategy outlined in Chart IV. It is not possible to selectively remove the C-terminal p-nitrobenzyl ester on intermediate 66 without disturbing some other portion of the molecule as well. Thus the activation and cyclization steps cannot be separated in the manner shown in Figure 53. This situation is often encountered when protected functional side chains appear in the acyclic precursors to cyclic peptides and depsipeptides. In these cases, the direct cyclization of peptides unprotected at both C- and N-terminal positions must be achieved and has often been reported.[1]



X = acid labile N-terminal protecting group

Figure 53. A General Activated Ester Cyclization Sequence.

However, separation of the activation and cyclization steps can be achieved for these free peptide derivatives under appropriate conditions. One example is activation by the formation of mixed carbonic anhydrides in a pyridinium chloride buffer.[110] This allows C-terminal activation while the amino group is protonated. Once the formation of the mixed anhydride is complete, the activated peptide can be cyclized by the addition of an appropriate base in dilute solution. A similar procedure for the separation of activation and cyclization for free peptides has been developed using Woodward's Reagent K.[111]

Alternatively, the cyclization of free peptides has been achieved by carrying out the activation step under conditions of high dilution by using a large excess of the activating reagents. In this method the activation and cyclization steps are not separated, but the activated acyclic peptide is formed only under conditions of high dilution. Intermolecular reactions leading to polymeric products are minimized.

The cyclization of free peptides by the formation of activated succinimido-esters under dilute conditions using excess N-hydroxysuccinimide and excess N,N'-dicyclohexylcarbodiimide (see Figure 40) is an example of this technique.[112-115] Although the risk of formation of unwanted side products exists for this method[100], it was chosen as a good method for initial attempts to cyclize octadepsipeptide 67 since it had been successfully applied to the cyclization

of a free peptide intermediate leading to isariin.[16]

The cyclization of octadepsipeptide 67 was attempted using this procedure (Figure 54).

A dilute solution of octadepsipeptide 67 (1.8×10^{-3} M) in a mixed methylene chloride-dimethylformamide solvent (34/1, v/v) was treated with 2.7 equivalents of N,N'-dicyclohexylcarbodiimide and 4.1 equivalents of N-hydroxysuccinimide (HOSu). After three days, the desired protected cyclic octadepsipeptide 68, norsurfactin di-tert-butyl ester, was obtained in 41% yield after purification of the crude reaction product by column chromatography on silica gel followed by high pressure gel permeation chromatography.

Removal of the side chain tert-butyl ester protecting groups on the aspartic and glutamic acid residues to afford norsurfactin was attempted using anhydrous trifluoroacetic acid (TFA) as shown in Figure 55. Norsurfactin (11) was obtained in 65% yield after purification by preparative layer chromatography on silica gel and high pressure liquid chromatography on C18/Porasil B. The infrared spectrum of the product was identical to the published spectra for surfactin.[20,22] The optical rotations of norsurfactin were: $[\alpha]_D^{25} = 27.1°$ (c 1, $CHCl_3$) and $[\alpha]_D^{25} = -35.2°$ (c 1, CH_3OH) while those reported for surfactin are: $[\alpha]_D^{27} = 40°$ (c 1, $CHCl_3$) and $[\alpha]_D^{27} = -39°$ (c 1, CH_3OH).[22] The differences in optical rotations between synthetic norsurfactin and surfactin might result from the difference in their structure, the presence of diastereomers in the synthetic compound, or

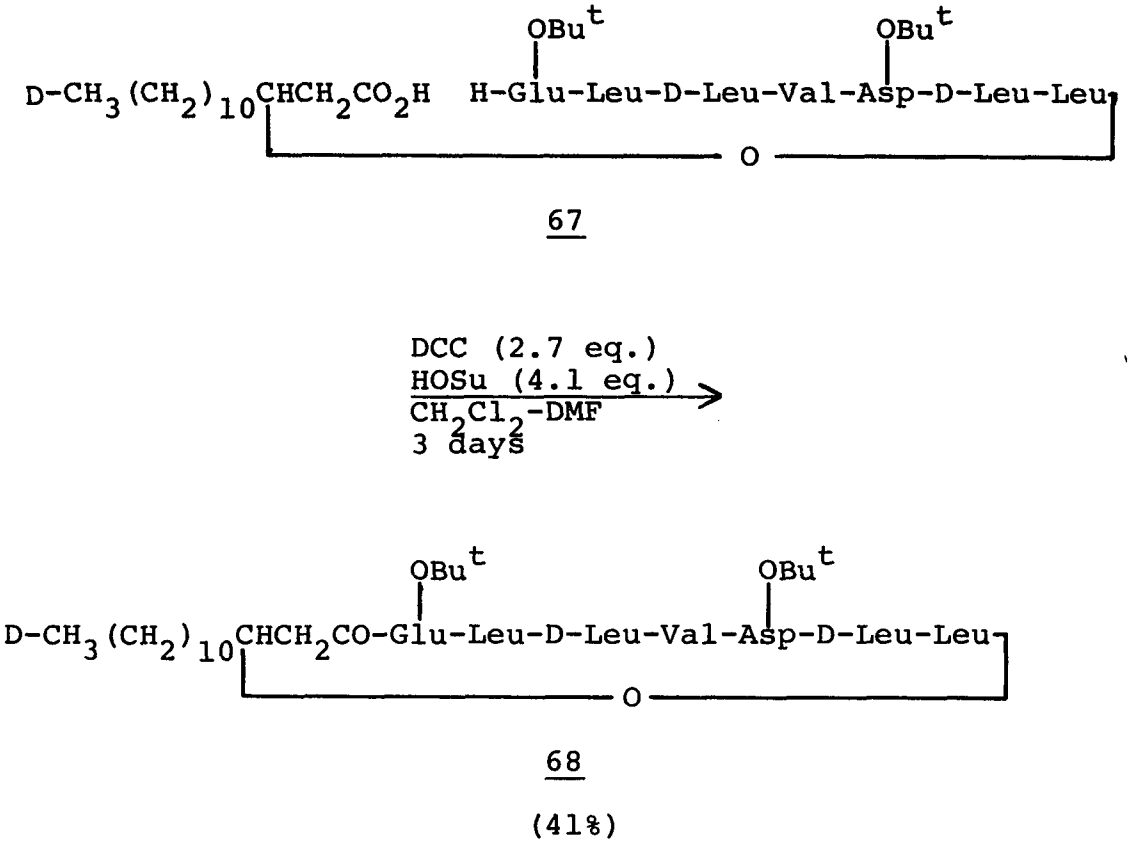
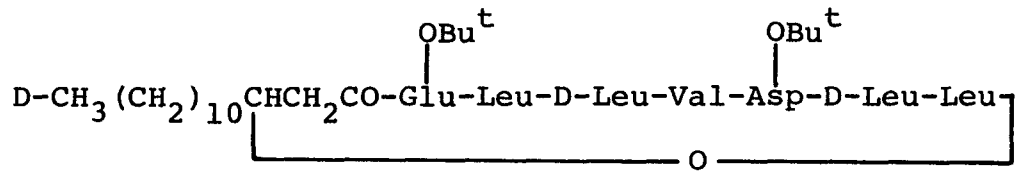
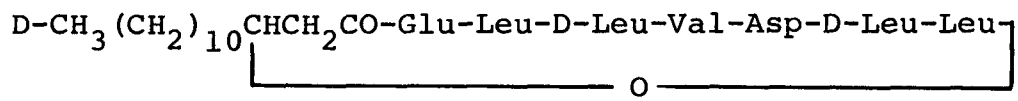


Figure 54. The Formation of the Protected Cyclo octadepsipeptide (69), Norsurfactin di-tert-Butyl Ester.



68

TFA →



11

(65%)

Figure 55. The Preparation of Norsurfactin (11).

the presence of impurities in natural surfactin. The elemental analysis for synthetic norsurfactin was determined to be: C, 60.77; H, 9.03; N, 9.30 while theoretical values are calculated as C, 61.09; H, 8.97; N, 9.59 for $C_{52}H_{91}N_7O_{13}$. The amino acid ratios of the synthetic compound were found to be: Asp, 0.99; Glu, 0.98; Leu, 4.05; Val, 0.94.

Experiments designed to test the biological activity* and the chemical purity of synthetic norsurfactin (11) are in progress.

Attempted cyclizations leading to a norsurfactin diastereomer (derived from model octadepsipeptide 65) are also being studied.

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*After the completion of this thesis, hemolytic activity data on synthetic norsurfactin was obtained. Under the conditions reported by Bernheimer and Avigad[20], synthetic norsurfactin exhibited hemolytic activity comparable to the activity exhibited by a sample of natural surfactin.

EXPERIMENTAL

General

Gas-Liquid Chromatography Analyses (GLC) were performed on a Varian Aerograph Model 90-P gas chromatograph coupled to a Sargent Welch Model SRG recorder with Disc integrator. A 3% SE-30 on Varaport 30 (80-100 mesh) column (5 ft. x 1/4 in.) was used for all analyses in this study. Helium was used as a carrier gas at a flow rate of 60 ml/min. Retention times and column temperature are indicated for each compound.

Infrared Spectra (ir) were recorded on a Perkin-Elmer 337 grating spectrophotometer and calibrated using the 1601.4 and 1028 bands of polystyrene. The spectra of liquids were obtained neat, while those of solids were obtained as mulls.

Nuclear Magnetic Resonance Spectra (nmr) were obtained on a Jeolco Model JNM-MH 100, 100 MHz nmr. All 100 MHz spectra are numbered less than 3000. A Varian Model A-60 Spectrometer was used to record 60 MHz nmr spectra. All 60 MHz spectra are numbered greater than 3000. Chemical shifts are reported relative to tetramethylsilane. Splitting patterns are designated as: s, singlet; d, doublet; t, triplet; ABq, AB quartet; m, multiplet.

Melting points were obtained using a Thomas-Hoover melting point apparatus and are uncorrected.

Optical Rotations were determined on a Carl Zeiss Photoelectric Precision Polarimeter.

Elemental Analyses were performed on an F&M Model 185 carbon, hydrogen and nitrogen analyzer by Ms. L. Heavner, Dr. G. Lambert and Ms. D. Cardin at the University of New Hampshire.

Amino Acid Analyses were performed on a Beckman model 120C amino acid analyzer.

High Pressure Liquid Chromatography was performed on a Waters ALC/GPC-202 chromatograph equipped with a model 6000 delivery system, USK injector and refractive index and ultra-violet detection systems.

Thin Layer Chromatography (TLC) was performed on silica gel (Adsorb. A) and basic alumina (Adsorb. B) plates in the following solvent systems:

A. Chloroform-acetone (80:20)

B. Chloroform-methanol-acetic acid (85:10:15)

C. Chloroform-acetone (90:10)

D. Ethanol-water (65:35)

E. n-Butanol-acetic acid-water-pyridine (15:3:12:10)

F. Pyridine-acetic acid-water (t0:30:15)

G. Chloroform-ethanol (80:20)

H. Acetonitrile-water (85:15)

I. Ethyl acetate-acetic acid-water (88:6:6)

J. Ethanol-28% ammonia water-water (7:3:2)

K. Methanol

Compounds were purchased from commercial sources and used as received, unless otherwise noted.

D,L-β-Hydroxytetradecanoic Acid (12). A mixture of zinc dust (18.5 g, 0.280 mol) and dodecylaldehyde (16) (50.5 g, 0.275 mol) in anhydrous tetrahydrofuran (100 ml) and trimethylborate (100 ml) was cooled to 10°. To this vigorously stirred cold mixture ethyl bromoacetate (17) (10 g portion) was added to initiate the reaction. When the vigorously exothermic reaction occurred the mixture was allowed to cool to room temperature and a dropwise addition of ethyl bromoacetate (total amount, 46.0 g, 0.275 mol) was resumed. When addition was completed the mixture was stirred at room temperature for 16 hr. Concentrated ammonium hydroxide (68 ml) and glycerine (68 ml) were added and stirring was continued for 30 min. The mixture was filtered and the filtrate was extracted with diethyl ether (3 x 300 ml). The ethereal extracts were combined, dried ($MgSO_4$) and concentrated to give crude D,L-ethyl-β-hydroxytetradecanoate (18) as a yellow oil.

A solution of potassium hydroxide (37.0 g) in 95% ethanol (400 ml) was added slowly to a stirred solution of oil 18 in 95% ethanol (100 ml). Stirring was continued overnight. The resulting mixture was diluted with distilled H_2O (2 ℓ) and washed with diethyl ether (2 x 500 ml). The aqueous phase was acidified with 10% H_2SO_4 and the resulting oil was extracted into diethyl ether (2 x 350 ml). The ethereal extracts were combined, dried ($MgSO_4$) and concentrated to a pale solid. The solid (45 g) was dissolved in benzene (150 ml), the hexane (450 ml) was added, and upon

cooling to -15° crystals formed. D,L-β-Hydroxytetradecanoic acid (12) was obtained as a white solid, 43.7 g (65%); mp 77-78°; lit.[82] mp 78-79°.

Resolution of D,L-β-Hydroxytetradecanoic Acid. To a gently boiling solution of D,L-β-hydroxytetradecanoic acid (12) (30.0 g, 0.123 mol) in anhydrous diethyl ether (325 ml) was added carefully a solution of (-)-α-methylbenzylamine, $[\alpha]_D^{25}$ -38.4° (neat); optical purity 96%; lit.[120] $[\alpha]_D^{25}$ -40.1° (neat), (14.8 g, 0.122 mol) in anhydrous diethyl ether (90 ml). After 12 hr at -15° the precipitated salt was filtered, washed well with diethyl ether and dried to give 41.4 g (93%) of the amine salt.

The salt was recrystallized from a mixture of anhydrous diethyl ether (414 ml) and absolute ethanol (207 ml) by cooling the solution for 6 days at 5°. The salt obtained (15.9 g) was recrystallized again from anhydrous diethyl ether (300 ml) and absolute ethanol (150 ml) by cooling the solution at 5° for 10 days to give 3.15 g (15%) of the amine salt. The rotation of the β-hydroxytetradecanoic acid obtained by decomposing a small amount of this salt with hydrochloric acid as in the procedure described below was: $[\alpha]_D^{25}$ -14.9° (c 2, $CHCl_3$), 93% ee. A second crop of amine salt (2.0 g, $[\alpha]_D^{25}$ -14.4, 90% ee) was obtained by cooling the mother liquors from prior crystallization and recrystallizing the precipitated salt (6.8 g) from anhydrous diethyl ether (125 ml) and absolute ethanol (63 ml).

The combined salts (5.00 g) were recrystallized from anhydrous diethyl ether (60 ml) and absolute ethanol (30 ml) by cooling the solution for 24 hr at 5° to give 3.35 g (16%) of the amine salt. This salt was shaken with a mixture of diethyl ether (100 ml) and 6N hydrochloric acid (100 ml). The ethereal layer was separated, washed with distilled H_2O (50 ml), dried ($MgSO_4$) and concentrated to a white solid. The solid was recrystallized from benzene (10 ml) by the addition of hexane (50 ml) with cooling to give (-)-D-β-hydroxytetradecanoic acid, 2.1 g (14.2%); mp 74-75°; $[\alpha]_D^{25}$ -15.4° (c 2, $CHCl_3$) (96% ee); lit.[82] mp 73-74°; $[\alpha]_D^{25}$ -16.0° (c 2, $CHCl_3$).

(+)-L-β-Hydroxytetradecanoic acid (12) was resolved in a similar fashion using the β-hydroxytetradecanoic recovered from the resolution of the D-enantiomer and (+)-α-methylbenzylamine. The desired L-β-hydroxytetradecanoic acid was obtained in 17% yield; mp 74-75°; $[\alpha]_D^{25}$ +15.4° (c 2, $CHCl_3$), 96% ee.

D,L-tert-Butyl-β-hydroxytetradecanoate (20). Dimethylformamide dineopentyl acetal (17.5 ml) was added to a solution of D,L-β-hydroxytetradecanoic acid (12) (4.4 g, 18 mmol) in anhydrous tert-butyl alcohol with stirring. The mixture was heated to 75° under reflux for 20 min. The solution was concentrated at 70° and the resulting oil was dissolved in diethyl ether (75 ml) and washed with H_2O (2 x 75 ml), saturated $NaHCO_3$ solution (2 x 75 ml), H_2O (75 ml) and 10% aqueous citric acid solution (75 ml). The

solution was then dried ($MgSO_4$) and concentrated to a yellow oil (6.12 g).

A sample of this oil (2.0 g) was purified by dry column chromatography on a 3 cm x 50 cm column of silica gel (Woelm) using benzene as the developing solvent. The product was extracted from the silica gel using diethyl ether and the extract was concentrated to a colorless oil, 1.2 g (60%). GLC analysis showed the oil to be greater than 97% D,L-tert-butyl-β-hydroxytetradecanoate, R.T. 2.25 min (col. temp. 170°). The retention time was identical to a previously prepared sample purified by preparative GLC; nmr spec. no. 16092 ($CDCl_3$), δ = 0.8 (t, 3H, $C\underline{H}_3$), 1.25 (s, 20H, 10 $-C\underline{H}_2-$), 1.4 (s, 9H, OBu^t), 2.3 (t, 2H, $-C\underline{H}_2-$), 3.6 (s, 1H, $-O\underline{H}$), 3.8 (m, 1H, $-\underset{|}{\overset{|}{C}}-\underline{H}$); ir spec. no. 20714 (neat) 3450 cm^{-1} (OH), 1725 (C=O), 1250 (C-H).

Anal. Calcd for $C_{18}H_{36}O_3$: C, 72.15; H, 12.00. Found: C, 72.38; H, 12.50.

Benzyloxycarbonyl-L-leucine (21). This compound was prepared in a manner analogous to that described for benzyloxycarbonyl-D-leucine (52) later in the experimental. A yield of 28.2 g (70%) of a highly viscous clear oil was obtained. TLC: Solv. A, Adsorb. A, R_f 0.78; $[\alpha]_D^{25}$ -15.6° (c 4.21, abs. EtOH); lit.[118] $[\alpha]_D^{25}$ -16.4° (abs. EtOH).

The Attempted Esterification of Benzyloxycarbonyl-L-leucine (21) with D,L-tert-Butyl-β-hydroxytetradecanoate (20). Method A. To a stirred solution of benzyloxycarbonyl-L-leucine (21) (0.87 g, 3.2 mmol) in anhydrous methylene

chloride (10 ml) at 0° a solution of N,N'-carbonyldiimidazole (0.52 g, 3.2 mmol) in anhydrous methylene chloride, was added dropwise, over a 15 min period. A solution of D,L-tert-butyl-β-hydroxytetradecanoate (20) (0.90 g, 3.0 mmol) in anhydrous methylene chloride (10 ml) was added and stirring was continued for 1 hr at 0° followed by 3 days at room temperature. The reaction mixture was then concentrated and the resulting oil was dissolved in diethyl ether (50 ml). The ethereal solution was washed with H_2O (20 ml), 10% citric acid solution (20 ml), H_2O (20 ml), 5% $NaHCO_3$ solution (2 x 20 ml) and H_2O (20 ml). The organic phase was dried ($MgSO_4$) and concentrated to a yellow oil, 0.79 g. GLC of this oil showed it to be greater than 90% D,L-tert-butyl-β-hydroxytetradecanoate (20).

Method B. The above procedure was repeated using tetrahydrofuran as the solvent. The reaction time was extended to 5 days and the reaction mixture worked up as before. Only D,L-tert-butyl-β-hydroxytetradecanoate was recovered from the reaction mixture.

Method C. A solution of benzyloxycarbonyl-L-leucine (21) (1.43 g, 5.4 mmol) in a mixture of anhydrous tetrahydrofuran (2.5 ml) and anhydrous pyridine (2.5 ml) was cooled to -15°. Benzenesulfonyl chloride (0.97 g, 5.4 mmol) was then added dropwise, with stirring, over 3 min. The resulting solution was stirred at -15° for 25 min. The solution was then cooled to -30° and a solution of D,L-tert-butyl-β-hydroxytetradecanoate (20) (1.15 g, 3.7 mmol) in anhydrous tetrahydrofuran (2 ml) was added. Stirring was continued at

-30° for 1 hr, at -10° for 1 hr, at 5° for 10 hr and at 25° for 2 hr. The reaction mixture was then poured into 1N HCl (15 ml) and then extracted with ethyl acetate (2 x 25 ml). The ethyl acetate extracts were combined and washed with 10% citric acid solution (15 ml), 5% $NaHCO_3$ solution (2 x 20 ml) and H_2O (20 ml). The organic phase was dried ($MgSO_4$) and concentrated to a dark brown oil, 2.1 g. GLC analysis of this oil showed it to be primarily D,L-tert-butyl-β-hydroxy-tetradecanoate (20) plus some unidentified volatile components. TLC (Solv. B, adsorb. B) indicated only starting tert-butyl ester (20) was present.

The Esterification of Benzyloxycarbonylglycine (23) with D,L-tert-Butyl-β-hydroxytetradecanoate (20). This compound was prepared by Method C as described for the attempted preparation of compound (22). After the initial workup the crude product was purified by preparative layer chromatography on silica gel using chloroform-acetone (97/3, v/v) as the developing solvent. Compound 24 was obtained as a clear oil, 0.36 g (75%); nmr spec. no. 1386 ($CDCl_3$) δ = 0.9 (t, 3H, $C\underline{H}_3$-), 1.3 (s, 20H, -$C\underline{H}_2$-), 1.5 (s, 9H, t-butyl-), 2.5 (d, 2H, -$C\underline{H}_2$-), 3.9 (d, 2H, -$C\underline{H}_2$- of Gly), 5.2 (s, 2H, -$C\underline{H}_2$-Ph), 5.3 (m, 1H, -$\underset{|}{\overset{|}{C}}$-$\underline{H}$), 5.9 (m, 1H, -N$\underline{H}$-), 7.4 (s, 5H, Ph-).

Anal. Calcd for $C_{28}H_{45}N_1O_6$: C, 68.55; H, 9.15; N, 2.85. Found: C, 68.49; H, 9.15; N, 2.76.

D,L-Methyl-β-hydroxytetradecanoate (25). A. Dimethylformamide dineopentyl acetal (20 ml) was added to a solution of D,L-β-hydroxytetradecanoic acid (12) (5.0 g, 20 mmol) in anhydrous methanol (100 ml) with stirring. The solution was refluxed for 20 min and then concentrated to a yellow oil to which H_2O (200 ml) was added. The resulting suspension was extracted into ethyl acetate (2 x 150 ml) and the combined extracts were washed successively with H_2O (2 x 100 ml), saturated $NaHCO_3$ solution (2 x 100 ml) and saturated NaCl (50 ml). The organic phase was dried ($MgSO_4$) and concentrated to a yellow oil. GLC analysis of this oil showed it to be 65% of the desired D,L-methyl-β-hydroxytetradecanoate (25), R.T. 2.6 min. The oil was dissolved in pentane (15 ml) and the solution was cooled to -15° for 2 days. The crystalline solid was collected and dried to give 0.90 g (17%) of pure D,L-methyl-β-hydroxytetradecanoate, mp 33-35°.

Method B. An ethereal solution of diazomethane was prepared from p-tolylsulphonyl-methyl-nitrosamide (21.5 g, 0.1 mol) according to the procedure of Vogel.[117] The ice-cold diazomethane solution was added slowly with stirring to an ice-cold suspension of D,L-β-hydroxytetradecanoic acid (12) (12.15 g, 50 mmol) in anhydrous diethyl ether (150 ml) until the yellow color of the solution persisted. A few drops of acetic acid were added to decompose any excess diazomethane and the mixture was concentrated to a yellow oil. This oil was dissolved in diethyl ether (75 ml) and washed with saturated $NaHCO_3$ solution (3 x 25 ml). The

organic phase was dried ($MgSO_4$) and concentrated to a colorless oil which solidified upon standing to give D,L-methyl-β-hydroxytetradecanoate (25) as a crystalline solid, 12.5 g (98%), mp 34-35°. GLC analysis showed the sample to be pure, R.T. 2.6 min (col. temp. 170°).

Anal. Calcd for $C_{15}H_{30}O_2$: C, 69.80; H, 11.62. Found: C, 69.85; H, 11.71.

The Attempted Esterification of Benzyloxycarbonyl-L-leucine (21) with D,L-Methyl-β-hydroxytetradecanoate (25). Method A. A solution of benzyloxycarbonyl-L-leucine (21) (1.46 g, 5.5 mmol) in a mixture of anhydrous tetrahydrofuran (4 ml) and anhydrous pyridine (4 ml) was cooled to -15°. Benzenesulfonyl chloride (0.98 g, 5.5 mmol) was then added dropwise, with stirring, over a 3 min period. Stirring was continued at -15° for 30 min and then the mixture was cooled to -30°. D,L-Methyl-β-hydroxytetradecanoate (25) (1.0 g, 3.75 mmol) was added and stirring was continued at -30° for 1 hr, at -15° for 1 hr, at 0° for 18 hr, and at room temperature for 24 hr. The reaction mixture was then poured into 3N HCl (30 ml) with stirring and the resulting mixture was extracted into ethyl acetate (2 x 40 ml). The combined estracts were washed successively with H_2O (2 x 50 ml), saturated $NaHCO_3$ solution (2 x 50 ml) and 3N HCl (50 ml). The organic phase was dried ($MgSO_4$) and concentrated to a colorless oil, 1.69 g. A sample of this oil (0.40 g) was purified by preparative layer chromatography on silica gel using 95% ethanol-H_2O (60/40, v/v) as the developing solvent.

Compound 26 was obtained as a crude oil, 0.11 g (25%).

Anal. Calcd for $C_{29}H_{41}N_1O_6$: C, 68.90; H, 9.37; N, 2.77. Found: C, 67.67; H, 9.78; N, 2.20.

Method B. To a stirred solution of benzyloxycarbonyl-L-leucine (21) (1.55 g, 5.85 mmol) in anhydrous methylene chloride (25 ml) at 0° was added a solution of N,N'-carbonyldiimidazole (0.95 g, 5.85 mmol) in anhydrous methylene chloride (15 ml), dropwise over a 30 min period. A solution of D,L-methyl-β-hydroxytetradecanoate (25) (1.5 g, 5.85 mmol) in anhydrous methylene chloride (10 ml) was then added. The reaction mixture was stirred at 0° for 1 hr and then at room temperature for 10 days. It was then concentrated and the resulting brown oil was dissolved in diethyl ether (50 ml) and was washed successively with H_2O (2 x 50 ml), 10% citric acid solution (2 x 50 ml), saturated $NaHCO_3$ solution (2 x 50 ml) and H_2O (50 ml). The organic phase was dried ($MgSO_4$) and concentrated to a yellow oil (2.36 g). This oil was dissolved in pentane (35 ml) and cooled at -15° for 24 hr. The D,L-methyl-β-hydroxytetradecanoate which precipitated was collected by filtration (0.13 g, mp 34-36°), and the filtrate was concentrated to a yellow oil. A sample of this oil (1.14 g) was purified by dry column chromatography on a 3.0 cm x 50 cm column of silica gel (Woelm) using 95% ethanol-H_2O (60/40, v/v) as the developing solvent. The product was extracted from the silica gel using ethyl acetate and the extract was concentrated to give crude compound 26 as a yellow oil, 0.71 g (40%).

Anal. Calcd for $C_{29}H_{47}NO_6$: C, 68.90; H, 9.37; N, 2.77. Found: C, 69.34; H, 9.86; N, 3.17.

The Esterification of Benzyloxycarbonylglycine (23) with D,L-Methyl-β-hydroxytetradecanoate (25). Method A. The esterification was carried out in a manner analogous to the esterification of benzyloxycarbonyl-L-leucine (21) with compound 25 (Method A). After work up of the reaction mixture, 1.60 g of a colorless oil was obtained. This oil was dissolved in pentane (25 ml) and the solution was cooled to -15° for 24 hr. The precipitated solid was collected and dried to afford compound 27, 1.18 g (70%); mp 43-44°; nmr spec. no. 1428 ($CDCl_3$) δ = 0.95 (t, 3H, CH_3-), 1.15 (s, 20H, $-CH_2-$), 2.6 (d, 2H, $-CH_2-$), 3.7 (s, 3H, $-OCH_3$), 4.0 (d, 2H, $-CH_2-NH-$), 5.25 (s, 2H, $-CH_2-Ph$), 5.4 (m, 2H, $-\overset{|}{C}-H$, $\geq N-H$), 7.5 (s, 5H, Ph-).

Anal. Calcd for $C_{25}H_{39}NO_6$: C, 67.40; H, 8.75; N, 3.12. Found: C, 67.61; H, 8.82; N, 3.16.

Method B. A procedure was used analogous to the procedure used for the esterification of benzyloxycarbonyl-L-leucine (21) with compound 25 (Method B). After work-up of the reaction mixture, 2.25 g of a yellow oil was obtained. This oil was dissolved in pentane (25 ml) and the solution was cooled to -15° for 24 hr. The precipitated solid was collected and dried to afford compound 27, 1.46 g, mp 41.5-42.5°. A second crop of solid was obtained by concentrating and cooling the mother liquors, 0.18 g, mp 42-43°. A total yield of 1.64 g (62%) of compound 27 was obtained.

D,L-Methyl-β-hydroxybutyrate (28). An ethereal solution of diazomethane was prepared from p-tolylsulfonyl-methyl-nitrosamide (43.0 g, 0.2 mmol) according to the procedure of Vogel.[117] The ice-cold diazomethane solution was added slowly, with stirring, to an ice-cold solution of D,L-methyl-β-hydroxybutyric acid (11.0 g, 0.105 mol) in anhydrous diethyl ether (100 ml) until the yellow color of solution persisted. A few drops of acetic acid were added to decompose any excess diazomethane and the mixture was concentrated to a yellow oil. The oil was distilled and the fraction boiling between 75-78° (25 mm) was collected to give 6.76 g (50%) of D,L-methyl-β-hydroxybutyrate (28). GLC analysis of the product showed it to be primarily a single component, R.T. 1.0 min (col. temp. 75°); nmr spec. no. 1331 ($CDCl_3$) δ = 1.26 (d, 3H, $C\underline{H}_3$-), 2.60 (d, 2H, -$C\underline{H}_2$-), 3.84 (s, 3H, -$OC\underline{H}_3$), 4.34 (m, 1H, -$\underset{|}{\overset{|}{C}}$-$\underline{H}$), 4.78 (s, 1H, -$O\underline{H}$).

The Esterification of Benzyloxycarbonylglycine (23) with D,L-Methyl-β-hydroxybutyrate (28). The esterification was carried out in a manner analogous to that described for the esterification of benzyloxycarbonyl-L-leucine (21) with compound 25 (Method A). After workup of the reaction mixture a yellow oil was obtained, 3.78 g. GLC analysis of this oil showed that it contained 74% of compound 29 corresponding to a 60% yield, R.T. 11 min (col. temp. 170°). A sample of this oil was purified by preparative layer chromatography on silica gel using chloroform as the developing solvent; nmr spec. no. 1339 ($CDCl_3$) δ = 1.2 (d, 3H, $C\underline{H}_3$-), 2.5 (t, 2H,

$-C\underline{H}_2-$), 3.6 (s, 3H, $-O-C\underline{H}_3$), 3.9 (d, 2H, $-C\underline{H}_2-NH-$), 5.2 (s, 2H, $-C\underline{H}_2-Ph$), 5.4 (m, 1H, $-\underset{|}{\overset{|}{C}}-\underline{H}$), 6.0 (d, 1H, $-N\underline{H}-$), 7.5 (s, 5H, Ph-).

Anal. Calcd for $C_{15}H_{19}NO_6$: C, 58.20; H, 6.20; N, 4.53. Found: C, 58.02; H, 6.24; N, 4.40.

The Esterification of Benzyloxycarbonyl-L-alanine (30) with D,L-Methyl-β-hydroxybutyrate (28). The esterification was carried out in a manner analogous to that described for the esterification of benzyloxycarbonyl-L-leucine (21) with compound 25 (Method A). After workup of the reaction mixture a yellow oil was obtained, 3.06 g. GLC analysis of this oil showed that it contained 84% of compound 31 corresponding to a 57% yield, R.T. 3 min (col. temp. 200°). A sample of this oil was purified by preparative layer chromatography on silica gel using chloroform as the developing solvent; nmr spec. no. 1338 ($CDCl_3$) δ = 1.2 (d, 3H, $C\underline{H}_3-$), 1.4 (d, 3H, $C\underline{H}_3-$), 2.6 (t, 2H, $-C\underline{H}_2-$), 3.6 (s, 3H, $-O-C\underline{H}_3$), 4.2 (m, 1H, $-C\underline{H}-N\underline{H}$), 5.2 (s, 2H, $-C\underline{H}_2-Ph$), 5.4 (m, 1H, $-\underset{|}{\overset{|}{C}}-\underline{H}$), 6.1 (d, 1H, $-N\underline{H}-$), 7.5 (s, 5H, Ph-).

Anal. Calcd for $C_{16}H_{21}NO_6$: C, 59.42; H, 6.54; N, 4.34. Found: C, 59.88; H, 6.58; N, 4.19.

The Esterification of Benzyloxycarbonyl-L-leucine (21) with D,L-Methyl-β-hydroxybutyrate (28). The esterification was carried out in a manner analogous to that described for the esterification of benzyloxycarbonyl-L-leucine (21) with compound 25 (Method A). After workup of the reaction mixture a yellow oil was obtained, 2.30 g. GLC analysis of

this oil showed that it contained 52% of compound 32 corresponding to a 34% yield, R.T. 17 min (col. temp. 175°). A sample of this oil was purified by preparative layer chromatography on silica gel using chloroform as the developing solvent; nmr spec no. 1368 ($CDCl_3$) δ = 1.0 (d, 6H, $2CH_3$-), 1.2 (d, 3H, CH_3-), 1.6 (m, 2H, $-CH_2$-), 3.7 (s, 3H, $-O-CH_3$), 4.2 (m, 1H, $-\overset{|}{C}-H$), 5.2 (s, 2H, $-CH_2-Ph$), 5.4 (q, 1H, $-CH-NH-$), 5.9 (d, 1H, $-NH-$), 7.5 (s, 5H, Ph-).

Anal. Calcd for $C_{19}H_{27}NO_6$: C, 62.55; H, 7.41; N, 3.84. Found: C, 62.81; H, 7.42; N, 3.75.

D-p-Nitrobenzyl-β-hydroxytetradecanoate (D-34). (-)-β-Hydroxytetradecanoic acid (9.66 g, 0.04 mol), triethylamine (4.0 g, 0.04 mol) and p-nitrobenzyl chloride (6.7 g, 0.04 mol) were dissolved in distilled ethyl acetate (100 ml) and stirred at reflux for 3 days. The precipitated triethylamine hydrochloride was removed by filtration and the filtrate was stirred with saturated NaCl solution (150 ml). The sodium salt of the unreacted hydroxyacid precipitated immediately and it was removed by filtration. The residue was washed with a small volume of acetone followed by diethyl ether. The filtrate and washings were combined and the organic layer was separated and washed successively with saturated $NaHCO_3$ solution (2 x 50 ml), distilled H_2O (50 ml), 10% citric acid solution (50 ml) and saturated NaCl solution (50 ml). The organic phase was dried ($MgSO_4$) and evaporated to a yellow oil which was immediately dissolved in pentane (250 ml) and allowed to crystallize at 5° for 24 hr. The

crystalline solid was filtered and allowed to dry, giving 10.37 g of a pale yellow solid, mp 55-56°. A second crop of 1.48 g (mp 54-56°) was obtained from the mother liquor by concentrating and cooling. The two crops were combined and recrystallized from ethyl acetate-pentane to give 11.4 g (77%) of a cream colored crystalline solid; mp 55-56°; $[\alpha]_D^{25}$ -12.5° (c 2, $CHCl_3$).

Anal. Calcd for $C_{21}H_{33}NO_5$: C, 66.47; H, 8.76; N, 3.69. Found: C, 66.64; H, 8.51; N, 3.74.

The sodium salt of the unreacted β-hydroxy acid isolated during the preparation of 34 was shaken with a mixture of 3N HCl (50 ml) and diethyl ether (50 ml). The $NaHCO_3$ washings from the workup of 34 were combined, acidified with 3N HCl and extracted into diethyl ether (50 ml). The ethereal phases were combined, dried ($MgSO_4$) and evaporated to a white solid which was recrystallized from a minimum volume of hot benzene by adding pentane and cooling to -15°. The precipitated D-β-hydroxytetradecanoic acid was collected and dried to give 1.63 g (17%), $[\alpha]_D^{25}$ -15.5 (c 2, $CHCl_3$).

L-p-Nitrobenzyl-β-hydroxytetradecanoate (L-34). Compound L-34 was prepared by the same procedure used for D-34. A total yield of 4.6 g (62%) was obtained (mp 54.5-55.5), $[\alpha]_D^{15}$ 11.1° (c 2, $CHCl_3$).

Anal. Calcd for $C_{21}H_{33}NO_5$: C, 66.47; H, 8.76; N, 3.69. Found: C, 66.84; H, 8.91; N, 3.71.

L-β-Hydroxytetradecanoic acid, 1.1 g (23%) was recovered during the workup procedure.

tert-Butoxycarbonyl-D-leucine Monohydrate (35). This compound was prepared by the procedure of Weinstein[93] as in the preparation of tert-butoxycarbonyl-L-leucine monohydrate (37), (see following description). A yield of 26.2 g (92%) of the crystalline monohydrate was obtained; mp 84-86°; $[\alpha]_D^{25}$ 24.8° (c 1, HOAc). TLC: Solv. A, Adsorb A, R_f 0.83; lit.[119] mp 86-87°, $[\alpha]_D^{25}$ 25.3° (c 1, HOAc).

tert-Butoxycarbonyl-leucine Monohydrate (37). A mixture of leucine (26.3 g, 0.20 mol) and 1,1,3,3-tetramethylguanidine (46.0 g, 0.40 mol) in dimethylformamide (500 ml) was cooled to 0° in an ice bath. To this cold stirred mixture was added tert-butylazidoformate (43.0 g, 0.30 mol) dropwise over a period of 30 min. The mixture was stirred first at 0° for 1 hr and then at room temperature for 3 days. A small amount of insoluble material was filtered and the filtrate was concentrated in vacuo (2 mm). The resulting oil was stirred with 20% citric acid solution (200 ml) and ethyl acetate (450 ml). The ethyl acetate phase was separated and the aqueous phase was extracted with ethyl acetate (2 x 50 ml). The ethyl acetate phases were combined and washed with distilled H_2O (3 x 75 ml) and saturated NaCl solution (50 ml). The organic phase was dried ($MgSO_4$) and concentrated to a clear oil. The crystalline monohydrate formed readily upon the addition of a few ml of distilled H_2O and was recrystallized from hot methylene chloride by the addition of pentane and cooling to give 42.3 g (85%) of a white crystalline solid; mp 83.5-85.5°; $[\alpha]_D^{25}$ -24.9° (c 1, HOAc);

TLC: Solv. A, Adsorb. A, R_f 0.81; lit.[119] mp 84°, $[\alpha]_D^{25}$ -24° (c 1, HOAc).

The Esterification of tert-Butoxycarbonyl-D-leucine (35) with L-p-Nitrobenzyl-β-hydroxytetradecanoate (L-34).

Method A. A solution of tert-butoxycarbonyl-D-leucine (35) (7.0 g, 2.9 mmol) in diethyl ether (50 ml) was dried over anhydrous $MgSO_4$ for 3 hr and then over anhydrous $CaSO_4$ for 3 hr. The solution was concentrated to a clear oil which was stored in vacuo over P_2O_5 for several hours.

A solution of this oil in anhydrous methylene chloride (50 ml) was cooled to 0° and a solution of N,N'-carbonyldiimidazole (4.7 g, 2.9 mmol) in anhydrous methylene chloride (25 ml) was added dropwise with stirring over a 30 min period. A solution of L-p-nitrobenzyl-β-hydroxytetradecanoate (L-34) (3.8 g, 1.0 mmol) in anhydrous methylene chloride (25 ml) was added and stirring was continued for 1 hr at 0° and then for 7 days at room temperature.

The solution was then concentrated and the oily residue was dissolved in diethyl ether (100 ml) and washed successively with distilled H_2O (2 x 100 ml), saturated $NaHCO_3$ solution (2 x 75 ml), distilled H_2O (100 ml), 10% citric acid solution (2 x 75 ml) and distilled H_2O (50 ml). The ethereal phase was then stirred with saturated $NaHCO_3$ solution (100 ml) overnight to remove the excess BOC-D-leucine (35). The organic phase was dried ($MgSO_4$) and concentrated to an oil which was further purified by filtration through a 3.5 x 13 cm column of silica gel using chloroform-acetone (80/20, v/v) as the eluant (250 ml collected). The

eluate was concentrated to a colorless oil, 5.27 g (90%); TLC: Solv. A, Adsorb. A, R_f 0.83; Solv. A, Adsorb. B, R_f 0.73; nmr spec. no. 16478 ($CDCl_3$) δ = 0.9 (d, 9H, $C\underline{H}_3$-), 1.2-1.4 (2s, m, 32-33H, $C\underline{H}_3$-, -$C\underline{H}_2$-, -$\underset{|}{\overset{|}{C}}$-$\underline{H}$), 2.5 (d, 2H, -$C\underline{H}_2$-), 4.0 (m, 1H, -$\underset{|}{\overset{|}{C}}$-$\underline{H}$), 5.0 (m, 3H, -$\underset{|}{\overset{|}{C}}$-H, -$C\underline{H}_2$-AR), 7.5 (ABq, 4H, AR-); $[\alpha]_D^{25}$ 6.7° (c 2.5, $CHCl_3$).

Anal. Calcd for $C_{32}H_{52}N_2O_8$: C, 64.85; H, 8.84; N, 4.73. Found: C, 65.33; H, 8.89; N, 4.81.

Method B. The same procedure was used as in method A with the following modifications; the reaction was run in anhydrous tetrahydrofuran solvent and was catalyzed by the addition of 2.5 ml of a solution of sodium (1.0 g) and imidazole (1.0 g) in anhydrous tetrahydrofuran (15 ml). The catalyst solution was added after the addition of compound L-34. The time of reaction was shortened to 3 days and work-up as in A gave a 97% yield of compound 36, $[\alpha]_D^{25}$ 7.3° (c 2, $CHCl_3$).

The Esterification of tert-Butoxycarbonyl-L-leucine (37) with D-p-Nitrobenzyl-β-hydroxytetradecanoate (D-34). The same procedure was used as was described for the preparation of compound 36 (Method A) using tert-butoxycarbonyl-L-leucine monohydrate (37) (22.4 g, 90 mmol) and D-34 (11.0 g, 29 mmol). The reaction was allowed to proceed for 5 days and upon workup gave compound 38, 13.0 g (76%); TLC: Solv. C, Adsorb. A, R_f 0.91; Solv. C, Adsorb. B, R_f 0.94; nmr spec no. 16630 ($CDCl_3$) δ = 0.9 (d, 9H, $C\underline{H}_3$-), 1.2-1.4 (2s, m, 32H, $C\underline{H}_3$-, - $C\underline{H}_2$, -$\underset{|}{\overset{|}{C}}$-$\underline{H}$), 2.6 (d, 2H, -$C\underline{H}_2$-), 4.2 (m,

1H, -$\overset{|}{C}$-H), 4.8-5.1 (s, m, 4H, -CH_2-AR, =N-H, -$\overset{|}{C}$-H), 7.6 (ABq, 4H, AR); $[\alpha]_D^{25}$ -7.8°.

Anal. Calcd for $C_{32}H_{52}N_2O_8$: C, 64.85; H, 8.84; N, 4.73. Found: C, 64.94; H, 9.09; N, 4.85.

The Preparation of Didepsipeptide 39. The same procedure was used as was used for the preparation of compound 40 (see following description). A yield of 3.8 g (92%) of compound 39 was obtained, TLC; Solv. B, Adsorb. A, R_f 0.47. Compound 39 was used without further purification for the preparation of compound 42.

The Preparation of Didepsipeptide 40. A solution of 38, (12.0 g, 20.3 mmol) and boron trifluoride diethyl etherate (8.14 ml, 61.0 mmol) in glacial acetic acid (150 ml) was stirred at room temperature for 30 min. The solution was then poured into 20% sodium acetate solution (800 ml) and solid $NaHCO_3$ was added until the mixture became basic (pH 8-9). The resulting oil was extracted into diethyl ether (3 x 250 ml) and the ethereal phases were combined and washed with distilled H_2O (2 x 500 ml). The ethereal solution was dried($MgSO_4$), decolorized (Norite A) and concentrated to a yellow oil (10.0 g, 100%). This oil was purified by dry column chromatography on three 3.5 x 35 cm silica gel (Woelm) columns using chloroform-methanol-acetic acid (85:10:5) as the developing solvent. The desired compound was extracted from the silica gel with ethyl acetate (200 ml per column). The ethyl acetate extracts were combined and concentrated to a volume of 100 ml and were washed with saturated $NaHCO_3$ solution (4 x 50 ml) and distilled H_2O (50 ml). The organic

phase was dried ($MgSO_4$) and concentrated to a yellow oil, 9.3 g (92%); TLC: Solv. B, Adsorb. A, R_f 0.61; nmr spec. no. 16630 ($CDCl_3$) δ = 0.90 (d, 9H, CH_3-), 1.1-1.4 (s, m. 23H, $-CH_2-$, $-\overset{|}{C}-H$), 2.6 (d, 2H, $-CH_2-$), 3.3 (m, 1H, $-\overset{|}{C}-H$), 4.8-5.1 (s, m, 3H, $-CH_2-AR$, $-\overset{|}{C}-H$), 7.5 (ABq, 4H, AR); $[\alpha]_D^{25}$ -0.51° (c 1.7, $CHCl_3$).

Anal. Calcd for $C_{27}H_{44}N_2O_6$: C, 65.82; H, 9.00; N, 5.69. Found: C, 65.90; H, 9.00; N, 5.85.

The Preparation of Tridepsipeptide 42. A solution of tert-butoxycarbonyl-D-leucine monohydrate (37), (4.66 g, 18.7 mmol) in anhydrous THF (100 ml) was dried over anhydrous $MgSO_4$ for 4 hr. The $MgSO_4$ was filtered off and the filtrate was added to a solution of 39 (9.2 g, 18.7 mmol) and 1-hydroxybenzotriazole monohydrate (5.75 g, 37.4 mmol) in anhydrous THF (100 ml). The resulting solution was cooled to 0° in an ice bath and then DCC (3.82 g, 19.0 mol) was added and the mixture was stirred at 0° for 1 hr, and then at room temperature for 2 hr. The precipitated DCU was filtered and washed well with THF. The filtrate and washings were combined and concentrated to a yellow oil which was dissolved in ethyl acetate (100 ml). The ethyl acetate solution was filtered to remove additional DCU and washed with distilled H_2O (2 x 100 ml), saturated $NaHCO_3$ solution (2 x 100 ml), 10% citric acid solution (100 ml), distilled H_2O (100 ml) and saturated NaCl solution (50 ml). The solution was dried ($MgSO_4$), decolorized (Norite-A), and concentrated to a volume of 25 ml. The oily solution was filtered through

a 4.5 x 15 cm column of basic alumina using ethyl acetate as the eluant (600 ml collected). The ethyl acetate was concentrated to a pale yellow oil, 13.2 g. The oil was dissolved in a mixture of chloroform-acetone (80:20, 15 ml) and filtered through a 4.5 x 15 cm column of silica gel (40-140 mesh) using chloroform-acetone (80:20) as the eluent (500 ml collected). The eluate was concentrated to a pale-yellow, viscous oil, 13.0 g (98%); TLC: Solv. A, Adsorb. B, R_f 0.89; $[\alpha]_D^{25}$ 0.3° (c 0.55, $CHCl_3$).

Anal. Calcd for $C_{38}H_{63}N_3O_9$: C, 64.65; H, 9.00; N, 5.95. Found: C, 64.67; H, 9.22; N, 6.00.

The Preparation of Tridepsipeptide 43. The same procedure was employed as was used for the preparation of compound 42 using tert-butoxycarbonyl-D-leucine monohydrate (3.4 g, 13.6 mmol) and compound 39 (6.6 g, 13.5 mmol). Compound 43 was obtained as a colorless oil, 9.2 g (97%); TLC: Solv. C, Adsorb. A, R_f 0.60; $[\alpha]_D^{22}$ -9.9° (c 2, $CHCl_3$).

Anal. Calcd for $C_{38}H_{63}N_3O_9$: C, 64.65; H, 9.00; N, 5.95. Found: C, 64.74; H, 9.31; N, 5.95.

The Preparation of Tridepsipeptide 44. A solution of compound 42,(1.5 g, 2.1 mmol) and boron trifluoride diethyl etherate (0.84 m, 6.3 mmol) in glacial acetic acid (15 ml) was stirred for 20 min at room temperature. The solution was poured into 20% sodium acetate solution (150 ml) and solid $NaHCO_3$ was added until the mixture became basic (pH 8-9). The resulting oil was extracted into diethyl ether (3 x 75 ml) and the ethereal phases were combined, washed with dis-

tilled H_2O (2 x 150 ml), dried ($MgSO_4$) and concentrated to a pale yellow oil, 1.19 g (93%); TLC: Solv. B, Adsorb. A, R_f 0.63; Solv. B, Adsorb. A, R_f 0.55; $[\alpha]_D^{25}$ -0.5° (c 3.01, $CHCl_3$).

Anal. Calcd for $C_{33}H_{55}N_3O_7$: C, 65.42; H, 9.15; N, 6.95. Found: C, 65.21; H, 9.12; N, 6.77.

The Preparation of Tridepsipeptide 45. The same procedure was employed as was used for the preparation of compound 44 but using compound 43 (2.0 g, 2.84 mmol) in place of 42. Compound 44 was obtained as a pale-yellow oil, 1.61 g (94%); TLC: Solv. B, Adsorb. A, R_f0.78; $[\alpha]_D^{25}$ 0.91° (c 2, $CHCl_3$).

Anal. Calcd for $C_{33}H_{55}N_3O_7$: C, 65.42; H, 9.15; N, 6.94. Found: C, 65.51; H, 9.16; N, 6.90.

Benzyloxycarbonyl(β-tert-butyl)aspartate (46). Compound 46 was prepared by the procedure of Schwyzer and Dietrich[103] and isolated as its dicyclohexylamine salt; mp 122.5-123°; $[\alpha]_D^{27}$ 6.10° (c 2, glacial HOAc); lit.[103] mp 125-126°; $[\alpha]_D^{25}$ 5.5° (c 1.72, 90% HOAc).

β-tert-Butyl Aspartate (47). Benzyloxycarbonyl-(β-tert-butyl)-aspartate dicyclohexylamine salt (46·DCHA), (2.5 g, 5.0 mmol) was stirred in a mixture of ethyl acetate (75 ml) and 10% citric acid solution (75 ml) for 30 min. The ethyl acetate phase was separated and the aqueous phase was extracted with ethyl-acetate (50 ml). The ethyl acetate phase and extracts were combined, dried ($MgSO_4$) and concentrated to a clear oil.

Palladium on carbon catalyst (5%, 0.4 g) was added to a degassed (N_2) solution of the above oil in methanol (50 ml) and glacial acetic acid (5 drops). The mixture was degassed again (N_2) and hydrogenated at 10 psi for 3 hr. The catalyst was removed by filtration through Celite and the filtrate was concentrated to a white solid. The solid was taken up in methanol (20 ml) and precipitated (as a gel) by the addition of anhydrous diethyl ether. The gel was filtered and dried in vacuo to give 0.45 g of a white solid. An additional 0.36 g was obtained from the mother liquors by concentrating them to a clear oil, adding diethyl ether to precipitate a gel and then drying in vacuo. Both solids were combined, triturated well with diethyl ether, and dried in vacuo overnight to give 0.80 g (84%) of a white solid; mp 188-190°d; TLC: Solv. B, Adsorb. A, R_f 0.15 $[\alpha]_D^{25}$ 8.1° (c 1.0, 90% acetic acid); lit.[105] mp 189-190°d, $[\alpha]_D^{25}$ 8.5° (c 1, 90% acetic acid).

o-Nitrophenylsulfenyl(β-tert-butyl)aspartate Dicyclohexylamine Salt (49). To a vigorously stirred solution of β-tert-butyl aspartate (47) (1.30 g, 6.8 mmol) in dioxane (17 ml) and 2N NaOH (3.4 ml) was slowly added o-nitrophenylsulfenyl chloride (48) (1.42 g, 7.5 mmol) over a period of 15 min as 2N NaOH (3.4 ml) was added to maintain the pH between 8 and 10. Distilled H_2O (75 ml) was added and the mixture was filtered. The filtrate was acidified to pH 5-6 by the addition of 0.5N H_2SO_4. The resulting oil was extracted into diethyl ether (3 x 75 ml). The ethereal phase was dried ($MgSO_4$) and concentrated to a volume of approximately 50 ml.

Dicyclohexylamine (1.25 ml) was added and the solution was placed in a freezer at -15° overnight. The precipitated salt was collected and dried, 1.63 g, mp 158-160°. An additional 0.74 g (mp 160-161°) was obtained from the mother liquors by concentrating and cooling. The combined solids were recrystallized from a minimum volume of methanol by the addition of diethyl ether with concentrating and cooling. A yield of 2.15 g (60%) of a bright yellow crystalline solid was obtained; mp 162-163°; TLC: Solv. D, Adsorb. A, R_f 0.95; $[\alpha]_D^{25}$ -51.7° (c 2, $CHCl_3$).

Anal. Calcd for $C_{26}H_{41}N_3O_6S$: C, 59.63; H, 7.89; N, 8.02. Found: C, 59.74; H, 7.76; N, 8.09.

The Preparation of Tetradepsipeptide 51. o-Nitrophenylsulfenyl(β-tert-butyl)aspartate dicyclohexylamine salt (49) (1.02 g, 1.95 mmol) was shaken with a mixture of diethylether (50 ml) and 10% citric acid solution (75 ml). The ethereal phase was separated, dried ($MgSO_4$) and concentrated to a bright yellow oil.

A solution of this oil, compound 50, (1.18 g, 1.95 mmol) and 1-hydroxybenzotriazole monohydrate (0.60 g, 2.90 mmol) in anhydrous THF (25 ml) was cooled to 0° in an ice bath and DCC (0.41 g, 2.0 mmol) was added. The mixture was stirred first at 0° for 1 hr and then at room temperature for 2 hr. The precipitated DCU was filtered and washed well with THF. The filtrate and washings were combined and concentrated to a yellow oil which was dissolved in ethyl acetate (100 ml).

The ethyl acetate solution was washed successively with distilled H_2O (2 x 50 ml), saturated $NaHCO_3$ solution (3 x 50 ml), 10% citric acid solution (50 ml), distilled H_2O (50 ml) and saturated NaCl solution (50 ml). The organic phase was dried ($MgSO_4$) and concentrated to a yellow oil. A solution of this oil in ethyl acetate (5 ml) was filtered through a 12 x 3.5 cm column of basic alumina using ethyl acetate as the eluant (200 ml collected). The ethyl acetate eluate was concentrated to a viscous oil which was dried in vacuo at 0.1 mm Hg at 60° overnight to give 1.25 g (70%) of a bright yellow glassy solid; TLC: Solv. A, Adsorb. A, R_f 0.95; Solv. B, Adsorb. A, R_f 0.91; $[\alpha]_D^{25}$ -34.4° (c 0.5, $CHCl_3$).

Anal. Calcd for $C_{47}H_{11}N_5O_{12}S$: C, 60.69; H, 7.69; N, 7.53. Found: C, 60.50; H, 7.86; N, 7.38.

Benzyloxycarbonyl-D-leucine (52). To a vigorously stirred mixture of D-leucine (25.0 g, 0.19 mol) and $NaHCO_3$ (36.4 g, 0.43 mol) in distilled H_2O (250 ml) was added benzyloxycarbonyl chloride (35.0 g, 0.206 mol) in 5 portions over 30 min. The mixture was stirred for 3 hr and the remaining insoluble material was removed by filtration. The solution was washed with diethyl ether (2 x 100 ml) and the aqueous phase was acidified to pH 5 by the addition of 6N HCl with stirring and cooling. The resulting oil was extracted into diethyl ether (3 x 200 ml) and the ethereal phases were combined and washed with distilled H_2O (2 x 150 ml) and saturated NaCl solution (100 ml). The ethereal solution was dried ($MgSO_4$) and concentrated to a viscous clear oil, 30.1 g (60%);

TLC: Solv. A, Adsorb. A, R_f 0.74; $[\alpha]_D^{25}$ 15.3° (c 4.83, abs. EtOH); lit.[118] (for the L derivative) $[\alpha]_D^{25}$ -16.4° (abs. EtOH).

L-Valine Methyl Ester Hydrochloride (53). A solution of valine (40 g, 0.34 mol) in anhydrous methanol (600 ml) was heated to reflux and a stream of dry HCl was bubbled through the reaction mixture. The addition of HCl was continued at reflux for 4 hr and the solution was then cooled to 0°. When the cold solution became saturated with HCl it was stoppered and placed in the refrigerator overnight. The solution was then concentrated to dryness and the residue was dissolved in methanol (100 ml) and concentrated to dryness again. This process was repeated three times and the residue was then dried in vacuo over KOH pellets. The product was recrystallized from methanol (250 ml) by the addition of diethyl ether (450 ml) and cooling to -15° to give 41.0 g (mp 172-173°) of a white crystalline solid. An additional 8.5 g (mp 171-172°) was obtained from the mother liquors by concentrating and cooling. Both crops were combined and recrystallized from methanol (150 ml) by the addition of diethyl ether (400 ml) to yield 47.6 g (82%) of a white crystalline solid; mp 172-173°; $[\alpha]_D^{25}$ +15.0° (c 2, H_2O); lit.[104] mp 170°, $[\alpha]_D^{25}$ +15.5° (c 2, H_2O).

Benzyloxycarbonyl-D-leucyl-valine Methyl Ester (54). A mixture of benzyloxycarbonyl-D-leucine (52) (25.0 g, 0.094 mol), valine methyl ester hydrochloride (53) (15.77 g, 0.094 mol), 1-hydroxybenzotriazole monohydrate (28.8 g, 0.188 mol) and N-methylmorpholine (9.5 g, 0.094 mol) in anhydrous THF

was cooled to 0°. DCC (20.6 g, 0.100 mol) was added and the mixture was stirred first at 0° for 1 hr and then at room temperature for 2 hr. The precipitated DCU was removed by filtration and washed well with THF. The filtrate and washings were combined and concentrated. The residue was dissolved in ethyl acetate (400 ml) and filtered to remove more DCU. The filtrate was washed successively with distilled H_2O (2 x 100 ml), saturated $NaHCO_3$ solution (2 x 100 ml), distilled H_2O (100 ml), 10% citric acid solution (2 x 100 ml), distilled H_2O (100 ml) and saturated NaCl solution (100 ml). The ethyl acetate solution was dried ($MgSO_4$) and concentrated to a yellow oil which solidified immediately. The solid was dissolved in ethyl acetate (100 ml) and filtered through a 4.5 x 15 cm column of basic alumina using ethyl acetate as the eluant (600 ml collected). The eluate was then concentrated to a volume of 150 ml, pentane (500 ml) was added, and the product was allowed to crystallize overnight. The product was filtered and dried to give 21.7 g of a white solid, mp 103-104.5°. An additional 7.5 g (mp 102-103.5°) was obtained from the mother liquor by concentrating and cooling. The resulting crude product was recrystallized from a minimum volume of ethyl acetate by the addition of 5 volumes of pentane. Total yield, 29.2 g (82%); TLC: Solv. A, Adsorb. A, R_f 0.87; $[\alpha]_D^{25}$ 32.7° (c 2, $CHCl_3$).

Anal. Calcd for $C_{20}H_{30}N_2O_5$: C, 63.47; H, 7.99; N, 4.70. Found: C, 63.40; H, 7.88; N, 7.44.

Benzyloxycarbonylleucyl-D-leucyl-valine Methyl Ester (56). Palladium on carbon catalyst (5%, 2.0 g) was added to a degassed solution (N_2) of benzyloxycarbonyl-D-leucyl-valine methyl ester (54) (28.0 g, 0.074 mol) in methanol (400 ml) containing distilled H_2O (10 ml) and glacial acetic acid (10 ml). The mixture was degassed again (N_2) and hydrogenated at 25 psi for 16 hr. The catalyst was removed by filtration through Celite and the filtrate was concentrated to a clear oil which was stored *in vacuo* for several hours to give the dipeptide methyl ester (55) as the acetate salt. The highly viscous oil obtained gave one ninhydrin positive spot on TLC analysis; R_f 0.38 (Solv. B, Adsorb. A).

A solution of this oil (23.1 g) in anhydrous THF (200 ml) was cooled to 0° and N-methyl morpholine (9.1 g, 0.091 mol) was added. To this stirred solution was added a solution of benzyloxycarbonyl-L-leucine (21) (20.0 g, 0.076 mol) and 1-hydroxybenzotriazole monohydrate (23.7 g, 0.155 mol) in anhydrous THF (100 ml). DCC (16.5 g, 0.080 mol) was then added and stirring was continued at 0° for 1 hr and then at room temperature for 2 hr. The precipitated dicyclohexylurea (DCU) was filtered and washed well with THF. The filtrate and washings were combined and concentrated to an oil which was dissolved in ethyl acetate (250 ml), filtered to remove additional DCU and washed successively with distilled H_2O (3 x 100 ml), saturated $NaHCO_3$ solution (3 x 75 ml), distilled H_2O (100 ml), 10% citric acid solution (2 x 75 ml), distilled H_2O (100 ml) and saturated NaCl solution (50 ml). The organic phase was dried ($MgSO_4$) and concen-

trated to a volume of approximately 50 ml. The solution was filtered through a 4.5 x 2.5 cm column of basic alumina using ethyl acetate as the eluant (700 ml collected). The eluate was concentrated to 50 ml and pentane (500 ml) was added. After 12 hr the crystalline solid was collected and dried, 19.8 g, mp 103-106°. An additional 2.0 g (mp 98-101°) was obtained by concentrating the mother liquors and crystallizing the resulting oily residue from ethyl acetate-pentane (1/10, v/v). Both solids were combined and recrystallized from ethyl acetate (75 ml) by the addition of pentane (400 ml) to give 14.1 g (39%) of a white crystalline solid, mp 119-120°; $[\alpha]_D^{25}$ 24.5° (c 2, $CHCl_3$). The mother liquors were concentrated to a clear oil which was purified by dry column chromatography on a 3 x 50 cm column of silica gel (Woelm) using chloroform-acetone (90/10, v/v) as the developing solvent. The desired tripeptide (56) was extracted from the silica gel with diethyl ether. The ethereal extract was concentrated to a volume of 15 ml, pentane (100 ml) was added, and the product was allowed to crystallize. The product was filtered and dried to give 4.9 g (13.5%) of a white solid, mp 108-109.5°, $[\alpha]_D^{25}$ 24.3° (c 2, $CHCl_3$). Total yield, 19.0 g (53%), TLC: Solv. B, Adsorb. A, R_f 0.73; Solv. C, Adsorb. A, R_f 0.58.

Anal. Calcd for $C_{26}H_{41}N_3O_6$: C, 63.52; H, 8.40; N, 8.55. Found: C, 63.13; H, 8.67; N, 8.51.

Leucyl-D-leucyl-valine Methyl Ester 57. Palladium on carbon catalyst (5%, 1.0 g) was added to a degassed solution (N_2) of benzyloxycarbonylleucyl-D-leucyl-valine methyl ester (56) (16.3 g, 33 mmol) in methanol (200 ml), distilled H_2O (5 ml) and glacial acetic acid (4 ml). The mixture was degassed again (N_2) and hydrogenated at 20 psi for 10 hr. The catalyst was removed by filtration through Celite and the filtrate was concentrated to an oily foam. The foam was dissolved in ethyl acetate (200 ml) and washed with saturated $NaHCO_3$ solution (2 x 25 ml) and distilled H_2O (50 ml). The ethyl acetate solution was dried ($MgSO_4$) and concentrated to a volume of 100 ml. Pentane (400 ml) was added and the product was allowed to crystallize at 0° for 24 hr. The product was collected and dried to give 7.7 g of a white cyrstalline solid, mp 142.5-143.5°. An additional 2.4 g (mp 142-143°) of product was obtained by concentrating and cooling the mother liquors. A total yield of 10.1 g (86%) was obtained; TLC: Solv. C, Adsorb. A, R_f 0.31; Solv. E, Adsorb. A, R_f 0.87; $[\alpha]_D^{25}$ 32.3° (c 2, CH_3OH).

Anal. Calcd for $C_{18}H_{35}N_3O_4$: C, 60.47; H, 9.87; N, 11.76. Found: C, 60.40; H, 9.87; N, 11.60.

Benzyloxycarbonyl(γ-tert-butyl)glutamate (58). Compound 58 was prepared by the procedure of Schwyzer and Kappeler[105] and was isolated as its dicyclohexylamine salt, mp 139-140.5°; $[\alpha]_D^{25}$ 6.6° (c 1.5, CH_3OH); lit.[105] mp 140-141°, $[\alpha]_D^{25}$ 7.2 ± 0.7 (c 1.452, CH_3OH).

Benzyloxycarbonyl(γ-tert-butyl)glutamyl-leucyl-D-leucyl-valine Methyl Ester (59). A solution of benzyloxycarbonyl-(γ-tert-butyl)-glutamate dicyclohexylamine salt (15.0 g, 29.0 mmol) in 50% ethanol (300 ml) was stirred with Dowex 50W-X8 ion exchange resin, H^+ form (29 ml, 55 meq) for 30 min. The resin was filtered and the filtrate was concentrated to a volume of 150 ml. The mixture was extracted with ethyl acetate (3 x 75 ml) and the extracts were combined and washed with distilled H_2O (50 ml). The ethyl acetate solution was dried ($MgSO_4$) and concentrated to give compound 58 as a colorless oil, 9.8 g (100%).

A solution of the above oil (9.8 g, 29 mmol), leucyl-D-leucylaline methyl ester (57) (10.0 g, 28.1 mmol) and 1-hydroxybenzotriazole monohydrate (8.7 g, 56 mmol) in anhydrous THF (250 ml) was cooled to 0° and DCC (5.85 g, 28.3 mmol) was added. The mixture was stirred first at 0° for 1 hr and then at room temperature for 2 hr. The precipitated DCU was filtered and washed well with THF. The filtrate and washings were combined and concentrated to a yellow oil which was dissolved in ethyl acetate (250 ml). The ethyl acetate solution was filtered to remove additional DCU and was then washed successively with distilled H_2O (2 x 100 ml), saturated $NaHCO_3$ solution (3 x 100 ml), distilled H_2O (100 ml), 10% citric acid solution (100 ml), distilled H_2O (100 ml) and saturated NaCl solution (50 ml). The organic phase was then dried ($MgSO_4$) and concentrated to a volume of 25 ml. The oily solution was filtered through a 4.5 x 20 cm column of

basic alumina using ethyl acetate as the eluant (650 ml collected). The ethyl acetate eluate was concentrated to 75 ml and pentane (300 ml) was added. A white gel which formed immediately was broken up and stirred overnight. The gel was filtered and dried *in vacuo* to give a white solid. The solid was triturated with a mixture of pentane and diethyl ether (80:20, 200 ml) and the gelatinous residue was filtered and dried *in vacuo* to give compound *59* as a white solid, 16.4 g (86%); mp 119-120°; TLC: Solv. A, Adsorb. A, R_f 0.68; Solv. B, Adsorb. A, R_f 0.68; $[\alpha]_D^{25}$ 5.8° (*c* 2, $CHCl_3$).

Anal. Calcd for $C_{35}H_{56}N_4O_9$: C, 62.10; H, 8.34; N, 8.28. Found: C, 61.99; H, 8.62; N, 8.37.

Amino acid ratio: Glu, 1.00; Leu, 1.99; Val, 1.01.

Attempted Preparation of Tetradepsipeptide 60.

Method A. A solution of compound *51* (1.20 g, 1.28 mmol) in anhydrous chloroform (7 ml) was cooled to 0°. A solution of HCl in chloroform (0.33N, 0.5 ml) was added with stirring and the solution was allowed to warm to room temperature over a 15 min period. The solution was then concentrated to a yellow oil which was dissolved in diethyl ether (30 ml) and cooled to -15° for several hours. None of the desired hydrochloride salt (*60·HCl*) precipitated and the solution was concentrated again to a yellow oil. The oil was triturated with pentane but this failed to produce a solid product. The TLC of this oil (Solv. A, Adsorb. A) indicated a ninhydrin positive component (R_f 0.00-0.10) as well as some residual compound *60* (R_f 0.52) and *o*-nitrophenylsulfenyl chloride (R_f

0.93). Attempts to purify the slower running component (R_f 0.00-0.10) by dry column chromatography on a 1.5 x 35 cm column of silica gel (Woelm) using chloroform-acetone (80/20, v/v) as the developing solvent led only to isolation of an oily mixture having several ninhydrin positive components, as revealed by TLC analysis (Solv. B, Adsorb. A).

A sample of this oil (0.53 g) was dissolved in diethyl ether (25 ml) and washed with saturated $NaHCO_3$ solution (2 x 25 ml), distilled H_2O (25 ml) and saturated NaCl solution (25 ml). The organic phase was dried ($MgSO_4$) and concentrated to a yellow oil (0.47 g); TLC; Solv. B, Adsorb. A, R_f 0.64 (ninhydrin +) and R_f 0.95 (ninhydrin +).

Anal. Calcd for $C_{41}H_{69}N_4O_{10}$: C, 63.37; H, 8.82; N, 7.21. Found: C, 57.60; H, 8.76; N, 6.30.

Method B. A solution of compound 51 (0.235 g, 0.26 mmol), ammonium thiocyanate (0.043 g, 0.55 mmol) and 2-methylindole (0.072 g, 0.55 mmol) in methanol-glacial acetic acid (1:1, 5 ml) was stirred for 4 hr at room temperature. The solution was then concentrated in vacuo and the only brown residue was dissolved in diethyl ether (25 ml) and washed with warm distilled H_2O (3 x 50 ml) and 2.5% NH_4OH solution (3 x 50 ml). The ethereal phase was dried ($MgSO_4$) and concentrated to a brown oil (0.20 g); TLC: Solv. A, Adsorb. A, R_f 0.93—identical to compound 51.

Method C. A solution of compound 51 (0.233 g, 0.25 mmol) and thiophenol (0.08 ml) in dimethylformamide (5 ml) was stirred for 3 hr at room temperature. The solution was

concentrated to an oily mixture; TLC analysis (Solv. A, Adsorb. A) showed the mixture to be primarily compound 51 (R_f 0.92).

Method D. A solution of compound 51 (0.117 g, 0.13 mmol) in ice cold 88% formic acid (7 ml) was stirred at 0° for 3 hr. Distilled H_2O (2.5 ml) was added and the mixture was concentrated to an oily residue. The residue was dissolved in ethyl acetate (15 ml) and washed with saturated $NaHCO_3$ solution (10 ml) and distilled H_2O (10 ml). The organic phase was dried and concentrated to a yellow oil which failed to yield a solid upon trituration with diethyl ether or with pentane. TLC analysis (Solv. F, Adsorb. A) indicated the oil was a mixture of several ninhydrin positive components.

Attempts to isolate compound 60 as a solid oxalate by addition of an ethereal solution of anhydrous oxalic acid to an ethereal solution of the above oil failed to yield a pure solid derivative.

Benzyloxycarbonyl(γ-tert-butyl)glutamyl-leucyl-D-leucyl-valine (61). A solution of NaOH (0.94N, 20 ml) was added to a stirred solution of tetrapeptide methyl ester 59 (4.0 g, 6.04 mmol) in 75% aqueous dioxane (120 ml). Stirring was continued for 35 min at room temperature and the solution was poured slowly into a vigorously stirred solution of ice-cold distilled H_2O (600 ml) containing HCl (1.18N, 17 ml). The precipitated solid was collected, washed well with distilled H_2O and dried *in vacuo* over KOH. It was then dissolved

in a mixture of chloroform and ethanol (80/20, v/v) and filtered through a 4.5 x 10 cm column of silica gel (40-140 mesh) using chloroform-ethanol (80/20, v/v) as the eluant (2 l collected). The eluate was concentrated to a gel which was triturated with pentane and dried *in vacuo* to give tetrapeptide 61 as a white amorphous solid, 2.84 g (71%); mp 120-130°; TLC: Solv. G, Adsorb. A, R_f 0.60; $[\alpha]_D^{25}$ 21.9° (*c* 2, $CHCl_3$).

Anal. Calcd for $C_{34}H_{54}N_4O_9$: C, 61.61; H, 8.21; N, 8.45. Found: C, 60.88; H, 8.26; N, 8.67.

α-Methyl-β-tert-butyl Aspartate Hydrochloride (62). Compound 62 was prepared by the procedure of Wünsch and Zwick[109] and was isolated as a white crystalline solid; mp 184-185°d; $[\alpha]_D^{23}$ 24.6° (*c* 2, abs. EtOH); lit.[109] mp 167°d, $[\alpha]_D^{25}$ 25.8 ± 0.5° (*c* 1.8, abs. EtOH).

Anal. Calcd for $C_9H_{15}NO_4 \cdot HCl$: C, 45.09; H, 7.57; N, 5.84. Found: C, 45.30; H, 7.66; N, 5.80.

Benzyloxycarbonyl(γ-tert-butyl-glutamyl)leucyl-D-leucyl-valyl(α-methyl-β-tert-butyl)aspartate (63). A mixture of tetrapeptide 61 (1.36 g, 2.04 mmol), α-methyl-β-tert-butyl aspartate hydrochloride (62) (0.49 g, 2.04 mmol) and 1-hydroxybenzotriazole monohydrate (0.62 g, 4.08 mmol) in anhydrous THF (20 ml) was cooled to 0°. To this stirred solution was added DCC (0.43 g, 2.08 mmol) followed by N-methyl morpholine (0.203 g, 2.06 mmol). Stirring was con-

tinued for 1 hr at 0° and then for 4 hr at room temperature. The reaction mixture was then filtered and the residue was washed well with THF. The filtrate and washings were combined and concentrated to dryness. The residue wad dissolved in warm ethyl acetate (75 ml) and washed successively with distilled H_2O (2 x 25 ml), saturated $NaHCO_3$ solution (2 x 25 ml), distilled H_2O (25 ml), 10% citric acid solution (2 x 25 ml) and distilled H_2O (25 ml). The organic phase was dried ($MgSO_4$) and concentrated to dryness. The residue was dissolved in a minimum volume of hot ethyl acetate and filtered through a 4.5 x 25 cm column of basic alumina using ethyl acetate as the eluant (700 ml collected). The eluate was concentrated to dryness and the residue was dissolved in warm ethyl acetate (35 ml). After standing at room temperature for 12 hr the insoluble material was filtered off and washed with ethyl acetate. The filtrate and washings were combined and concentrated to dryness and the residue was triturated with pentane to give compound 63, 1.11 g (65%); mp 186-190°; TLC: Solv. A, Adsorb. A, R_f 0.61; $[\alpha]_D^{25}$ 18.1° (c 2, $CHCl_3$).

Anal. Calcd for $C_{43}H_{69}N_5O_{12}$: C, 60.90; H, 8.20; N, 8.26. Found: C, 60.84; H, 8.15; N, 8.19.

Amino acid ratio: Asp, 1.02; Glu, 1.03; Leu, 1.94; Val, 1.00.

Benzyloxycarbonyl(γ-tert-butyl)glutamyl-leucyl-D-leucyl-valyl(β-tert-butyl)aspartate (64). A solution of NaOH (0.94N, 6.77 ml) was added to a stirred solution of penta-

peptide 63 (1.74 g, 2.05 mmol) in 75% aqueous dioxane (60 ml). The solution was stirred for 23 min at room temperature and was then poured slowly into a vigorously stirred solution of ice-cold distilled H_2O (250 ml) containing HCl (1.18N, 5.5 ml). The precipitated solid was collected, washed well with distilled H_2O and dried in vacuo over KOH pellets.

The resulting solid was dissolved in a minimum amount of chloroform-ethanol (80/20, v/v) and the solution was filtered through a 4.5 x 25 cm column of silica gel (40-140 mesh) using chloroform-ethanol (80/20, v/v) as the eluant (2 L collected). The eluate was concentrated to a gel-like solid which was triturated with pentane and dried to give compound 64 as a white solid, 1.40 g (82%); mp 179-181°; TLC: Solv. G, Adsorb. A, R_f 0.59; $[\alpha]_D^{25}$ -19.2° (c 2, $CHCl_3$).

Anal. Calcd for $C_{42}H_{67}N_5O_{12}$: C, 60.48; H, 8.10; N, 8.40. Found: C, 60.22; H, 8.15; N, 8.67.

The Preparation of Octadepsipeptide 65. The same procedure was employed as was used for compound 66 (see following description) using pentapeptide 64 (0.746 g, 0.894 mmol) and tridepsipeptide 44 (0.542 g, 0.894 mmol). Compound 65 was obtained as a white solid, 0.71 g (60%); mp 175-178°; TLC: Solv. A, Adsorb. A, R_f 0.87; $[\alpha]_D^{25}$ 2.1° (c 2, $CHCl_3$).

Anal. Calcd for $C_{75}H_{120}N_8O_{18}$: C, 63.35; H, 8.51; N, 7.88. Found: C, 63.05; H, 8.43; N, 7.99.

Amino acid ratio: Asp, 1.01; Glu, 0.99; Leu, 3.99; Val, 1.01.

The Preparation of Octadepsipeptide 66. A solution of pentapeptide 64 (0.501 g, 0.60 mmol), tridepsipeptide 45 (0.364 g, 0.60 mmol) and 1-hydroxybenzotriazole monohydrate (0.184 g, 1.20 mmol) in anhydrous THF (50 ml) was cooled to 0° with stirring. DCC (0.128 g, 0.62 mmol) was then added and stirring was continued at 0° for 1 hr and at room temperature for 5 days. The reaction mixture was then filtered and the residue was washed well with THF. The filtrate and washings were combined and concentrated to dryness. The residue was dissolved in ethyl acetate (200 ml) and washed successively with distilled H_2O (2 x 100 ml), saturated $NaHCO_3$ solution (2 x 100 ml), 10% citric acid solution (2 x 100 ml), distilled H_2O (100 ml) and saturated NaCl solution (100 ml). The organic phase was dried ($MgSO_4$) and concentrated to a gelatinous residue which was dissolved in a minimum volume of warm ethyl acetate and filtered through a 4.5 x 25 cm column of basic alumina using ethyl acetate as the eluant (800 ml collected). The eluate was concentrated to a gelatinous residue which was dissolved in a minimum volume of ethyl acetate. The solution was allowed to stand and the insoluble material was filtered. The filtrate was concentrated to a gel which was triturated well with pentane and dried. The solid was reprecipitated from hot ethyl acetate (25 ml) by the addition of pentane (200 ml). The resulting gel was collected and dried to give compound 66 as a white solid 0.64 g (75%); mp 154-156°; TLC: Solv. A, Adsorb. A, R_f 0.75; $[\alpha]_D^{25}$ 30.1° (c 2, $CHCl_3$).

Anal. Calcd for $C_{75}H_{120}N_8O_{18}$: C, 63.35; H, 8.51; N, 7.88. Found: C, 63.26; H, 8.40; N, 7.90.

Amino acid ratio: Asp, 1.00; Glu, 1.00; Leu, 3.97; Val, 1.03.

The Preparation of Octadepsipeptide 67. Palladium on carbon catalyst (5%, 0.05 g) was added to a degassed solution (N_2) of compound 66 (0.50 g, 0.35 mmol) in methanol (35 ml). The solution was degassed again (N_2) before hydrogenating at atmospheric pressure for 12 hr. The catalyst was then removed from the mixture by filtration through Celite and the filtrate was concentrated to dryness. The residue was triturated repeatedly with pentane (6 x 50 ml) and dried to give compound 67 as a white solid, 0.380 g (94%); mp 134-137 ; TLC: Solv. H, Adsorb. A, R_f 0.87; Solv. G, Adsorb. A; R_f 0.90, $[\alpha]_D^{25}$ 51.0° (c 1, $CHCl_3$).

Anal. Calcd for $C_{60}H_{109}N_7O_{14}$: C, 62.52; H, 9.53; N, 8.51. Found: C, 61.85; H, 9.86; N, i.53.

The Preparation of Cyclooctadepsipeptide 68. A solution of compound 67 (0.369 g, 0.320 mol) in anhydrous dimethylformamide (5.0 ml) was diluted with anhydrous methylene chloride (170 ml). N-Hydroxysuccinimide (0.150 g, 1.30 mmol) was added with stirring and the solution was cooled to 0°. DCC (0.181 g, 0.88 mmol) was added and stirring was continued for 6 hr at 0° and then for 3 days at room temperature. The solution was then concentrated to dryness. The residue was dissolved in ethyl acetate (5 ml) and concentrated to dryness again. This process

was repeated twice and the resulting residue was purified by column chromatography on a 4 x 45 cm column of silica gel (100-200 mesh) using chloroform-acetone (80/20, v/v) as the eluant. The fractions containing the desired product (R_f 0.64, TLC Solv. A, Adsorb. A) were combined and concentrated to dryness.

The residue was dissolved in ethyl acetate (0.25 ml) and pentane (20 ml) was added. The solution was cooled to -15° for several hours. The precipitated solid was collected and dried to give 0.190 g (52%) of a white solid. This solution was purified further by high pressure gel permeation chromatography on a 4 ft x 3/8 in. Poragel 60 Å (Waters) column using ethyl acetate as the eluant. The fractions having a retention volume of 24 ml were collected, pooled and concentrated to dryness. The residue was crystallized from ethyl acetate (0.25 ml) by the addition of pentane (20 ml) with cooling to give the protected cyclooctadepsipeptide 68 as a white solid, 0.149 g (40%); mp 129-131°; TLC: Solv. A, Adsorb. A, R_f 0.65; $[\alpha]_D^{25}$ -25.4° (c 1, $CHCl_3$), ir spec. no. 22111 (nujol) 3300 cm^{-1} (N-H), 1735, 1720 (C=O, ester, lactone), 1650 (amide I), 1535 (amide II).

Anal. Calcd for $C_{60}H_{107}N_7O_{13}$: C, 63.52; H, 9.51; N, 8.64. Found: C, 63.79; H, 9.56; N, 8.78.

Amino acid ratio: Asp, 0.99; Glu, 0.98; Leu, 4.05; Val, 0.94.

Norsurfactin (11). A solution of compound 68 (90 mg, 0.079 mmol) in anhydrous trifluoroacetic acid (0.7 ml) was stirred for 30 min at room temperature. The solution was concentrated and the residue was stored in vacuo over KOH for several hours. It was purified by preparative layer chromatography on three 20 x 20 cm silica gel plates using ethyl acetate-acetic acid-water (18:6:6). The product (R_f 0.8-0.85) was extracted from the silica gel using ethyl acetate. The extract was concentrated and the residue was further purified using high pressure liquid chromatography on a 4 ft. x 3/8 in. C_{18}/Porasil B (Waters) column using acetonitrile-water (80:20) as the solvent. The product with retention volume 735 ml (after 2 recycles) was collected. After concentration of the solvent the resulting residue was dissolved in ethyl acetate and precipitated by the addition of pentane. The solid product was collected and dried to give norsurfactin, 51 mg (65%); mp 138-142°; $[\alpha]_D^{25}$ 27.1° (c 1, $CHCl_3$), -35° (c 1, CH_3OH); ir spec. no. 22117 (KBr) 3200-3500 cm^{-1} (N-H, O-H), 1735 (C=O, lactone), 1660 (amide I), 1540 (amide II); TLC*: Solv. H, Adsorb. A, R_f 0.43; Solv. I, Adsorb. A, R_f 0.80; Solv. J, Adsorb. A, R_f 0.81; Solv. K, Adsorb. A, R_f 0.73.

Anal. Calcd for $C_{52}H_{91}N_7O_3$: C, 61.09; H, 8.97; N, 9.59. Found: C, 60.77; H, 9.03; N, 9.30.

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*R_f values given are for the major component. In some solvent systems minor impurities having lower R_f values were still evident.

Amino acid ratio: Asp, 0.97; Glu, 1.01; Leu, 4.05; Val, 0.99.

Lit.[22] for surfactin; mp 140-141°; $[\alpha]_D^{27}$ 40° (c 1, $CHCl_3$), -39° (c 1, CH_3OH), TLC: Solv. I, Adsorb. A, R_f 0.8; Solv. J, Adsorb. A, R_f 0.8; Solv. K, Adsorb, A, R_f 0.7.

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